PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

EDGAR B. CAHOON ET AL.

CASE NO: BB1295 US CNT

SERIAL NO: 10/690,994

GROUP ART UNIT: 1638

FILED: OCTOBER 21, 2003

EXAMINER: LI ZHENG

FOR: PLANT DIACYLGLYCEROL ACYLTRANSFERASES

APPEAL BRIEF

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Commissioner for Patents
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Sir:

Pursuant to 37 C.F.R. § 1.192, the following is an Appeal Brief in support of the Appeal filed February 8, 2008, appealing the Final Office Action dated August 8, 2007. Submitted herewith is the filing fee for this Appeal Brief in accordance with 37 C.F.R. § 41.20(b)(2). Please charge said fee to Deposit Account No. 04-1928 (E.I. du Pont de Nemours and Company).

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I. REAL PARTY IN INTEREST

The real party in interest is E.I. du Pont de Nemours and Company (*hereinafter* "DuPont"), owner of the Application.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Applicants, Applicants' legal representative, or DuPont that will directly affect or be directly affected by or have a bearing on the Board of Patent Appeals and Interferences' (*hereinafter* the "Board") decision in the present Appeal.

III. STATUS OF THE CLAIMS

Claims 26 and 30-40 stand rejected and are the subject of this Appeal. Originally-filed claims 1-25 and 27-29 have been canceled.

IV. STATUS OF AMENDMENTS

In response to the Final Office Action, Applicants amended claim 26 to change the percent identity in part (a) from 90% to 95%. Claim 29 was canceled. As of the filing date of this Appeal Brief, the Examiner has not acted upon the amended claim or the canceled claim.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 26, the only independent claim, relates to an isolated polynucleotide comprising: (a) a nucleotide sequence encoding a polypeptide having diacylglycerol acyltransferase activity (*see, e.g.*, Applicants' Specification at page 1, lines 25-26; page 3, lines 9-10), wherein the polypeptide has an amino acid sequence of at least 95% sequence identity when compared to SEQ ID NO:16 (*see, e.g.*, Applicants' Specification at page 8, lines 4-6) based on the Clustal alignment method with pairwise alignment default parameters of KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5 (*see, e.g.*, Applicants' Specification at page 8, lines 12-16), or (b) a full-length complement of the nucleotide sequence of (a) (*see, e.g.*, Applicants' Specification at page 5, lines 25-28).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 26 and 30-40 have utility under 35 U.S.C. § 101.

Whether claims 26 and 32-40 are supported by sufficient written description under 35 U.S.C. § 112, 1st Paragraph.

Whether claims 26 and 30-40 are enabled under 35 U.S.C. §112, 1st paragraph, because the claimed invention is allegedly not supported by either a specific and/or substantial asserted utility or a well-established utility.

Whether claims 26 and 30-40 are enabled under 35 U.S.C. § 112, 1st Paragraph.

VII. ARGUMENT

A. Claims 26 and 30-40 Have Sufficient Utility as Required by 35 U.S.C. § 101.

In the August 8, 2007, Final Office Action, Claims 26 and 30-40 were rejected under 35 U.S.C. § 101 as not being supported by either a specific and/or substantial assorted utility or a well-established utility.

For an invention to have utility, "at least one specific, substantial, and credible utility" must be either disclosed in the specification or well-established for the invention. Utility Examination Guidelines, 66 Fed. Reg. 1092, 1094 (Jan. 5, 2001). Based upon this standard, Applicants respectfully submit that the present rejection under 35 U.S.C. § 101 for lack of utility is improper because at least one specific, substantial, and credible utility for the present application was asserted or is well-established. The standard for utility is a low one, with the MPEP noting that any utility beyond a "throw-away", "insubstantial", or "nonspecific" utility is sufficient to satisfy the utility requirement of 35 U.S.C. 101. MPEP § 2107(II)(B)(1)(i). The instant disclosure fulfills this low standard, as here Applicants have asserted diacylglycerol acyltransferase (DGAT) activity as a utility of the present invention.

The Office rejected the claims as lacking this asserted utility. "To properly reject a claimed invention under 35 U.S.C. 101, the Office must (A) make a *prima facie* showing that the claimed invention lacks utility, and (B) provide a sufficient evidentiary basis for factual assumptions relied upon in establishing the *prima facie* showing." MPEP § 2107.02(IV) (citing *In re Gaubert*, 524 F.2d 1222, 1224, 18

USPQ 664, 666 (CCPA 1975)). Applicants respectfully submit that the Examiner's evidence fails to establish a *prima facie* case of lack of utility. The Examiner's evidence consists of (1) citations to irrelevant journal articles and (2) an incorrect conclusion that Applicants failed to provide an assay for determining DGAT activity. Regarding the journal articles, several of these references relate to the dangers of relying on sequence comparisons for determining the activity of a protein. December 18, 2006, Non-Final Office Action, at 3-4 (*hereinafter* "NF Office Action"). First, novel gene sequences routinely have their function identified correctly by sequence comparison. Second, the USPTO uses sequence homology to search the prior art. Third, in the particular case of DGAT, four "putative" DGAT proteins from human, Arabidopsis, soybean, and wheat were subsequently shown to all have the expected DGAT activity. Consequently, for Applicants' protein of interest, the validity of sequence comparison has been documented.

The remaining references cited in the NF Office Action relate to determining the activity of enzymes that modify fatty acids based on sequence identity to known proteins. NF Office Action, at 4-5. The cited references, however, do not relate to determining DGAT activity from previously known sequences, and the Examiner provides no evidence that the difficulties set forth in those references are applicable to DGATs.

Regarding the second basis of the Examiner's utility rejection, the Examiner stated that "the specification does not provide any additional information, such as an enzyme assay, to establish the utility of the claimed sequences." *Id.* at 3 (emphasis added). Applicants, however, provided in Example 8 of their Specification a citation to a journal article containing a DGAT assay, Andersson *et al.*, J. Lipid. Res. 35:535-45 (1994). The assay in Andersson *et al.* was developed to assay DGAT purified from rat livers. Briefly, rat livers are isolated, homogenized, and microsomes are purified. Microsomes are then treated with detergent, sonication, and filtration to obtain enzyme preparations. 1,2-Dioleoyl-sn-glycerol and radioactively-labeled palmitoyl-CoA are used as substrates. The triacylglycerol product is purified by thin-layer chromatography, and the amount is measured by use of a liquid scintillator. Because it is well-established that an applicant need not disclose that which is known in the art, *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed.

Cir. 1991), Applicants' citation of this reference in the specification should be sufficient evidence of a well-established utility, that is DGAT activity, and therefore the Examiner's failure to establish a *prima facie* case of lack of utility.

Even if the Examiner established a *prima facie* case of lack of utility, Applicants provided sufficient evidence showing that the asserted utility is substantial, specific, and credible. If an examiner meets the burden of establishing a *prima facie* case of lack of utility, "the burden of coming forward with evidence or argument shifts to the applicant. . . . After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of evidence with due consideration to persuasiveness of argument." *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992); *see also In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288, 297 (CCPA 1974) ("Assuming that sufficient reason to question the statement and its scope does exist, a rejection for lack of utility under § 101 will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the statement of utility and its scope as found in the specification are true.").[1] The utility requirement for inventions relating to nucleic acid sequences can be satisfied by basing an asserted use "upon homology to existing nucleic acids or proteins having an accepted utility." Utility Examination Guidelines, 66 Fed. Reg. 1092, 1096 cmt. 19 (Jan. 5, 2001).

> When a class of proteins is defined such that the members share a specific, substantial, and credible utility, the reasonable assignment of a new protein to the class of sufficiently conserved proteins would impute the same specific, substantial, and credible utility to the assigned protein. If the preponderance of the evidence of record, or of sound scientific reasoning, casts doubt upon such an asserted utility, the examiner should reject the claim for lack of utility under 35 U.S.C. 101. For example, where a class of proteins is defined by common structural features, but evidence shows that the members of the class do not share a specific, substantial functional attribute or utility, despite having structural features in common, membership in the class may not impute a specific, substantial, and credible utility to a new member of the class.

[1] Under a preponderance of the evidence standard, "evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true." MPEP § 2107.02(VII) (emphasis in original).

Id. As detailed below, Applicants' evidence demonstrates, by at least a preponderance of the evidence, that the DGAT activity of the claimed sequences is a substantial, specific, and credible utility.

Substantial Utility

Applicants' evidence establishes that DGAT activity is a "substantial" activity. "Courts have used the labels 'practical utility' and 'real world' utility interchangeably in determining whether an invention offers 'substantial' utility." *In re Fisher*, 421 F.3d 1365, 1371, 76 USPQ2d 1225, 1230 (Fed. Cir. 2005). "'Practical utility' is a shorthand way of attributing 'real-world' value to claimed subject matter. In other words, one skilled in the art can use a claimed discovery in a manner which provides some immediate benefit to the public." *Nelson v. Bolwer*, 626 F.2d 853, 856, 206 USPQ 881, 883 (CCPA 1980). As noted in the specification, "DGAT plays a fundamental role in the metabolism of glycerolipids" (page 1, line 15). Further, "DGAT is important for the generation of seed oils . . ." (page 1, line 21). The product of the DGAT reaction is triacylglycerol, and "[i]n eukaryotic cells triacylglycerols are quantitatively the most important storage form of energy" (page 1, lines 11-12).

Further, Applicants note that DGAT activity does not fall into the categories of situations requiring further research to establish a substantial utility. The MPEP sets forth the following examples of insubstantial utilities:

> (A) Basic research such as studying the properties of the claimed product itself or the mechanisms in which the material is involved; (B) A method of treating an unspecified disease or condition; (C) A method of assaying for or identifying a material that itself has no specific and/or substantial utility; (D) A method of making a material that itself has no specific, substantial, and credible utility; and (E) A claim to an intermediate product for use in making a final product that has no specific, substantial and credible utility.

MPEP § 2107.01(I)(B) (emphasis in original). DGAT activity is not a study of the basic properties of the claimed sequences; it is not a method of treating an unspecified disease; it is not a method of identifying another material; it is not method of making a material; and it is not an intermediate product. While not being in one of these examples is not conclusive evidence of substantial utility, Applicants

believe that DGAT activity is so far removed from the insubstantial nature of these examples to question how the Examiner can consider DGAT activity to be insubstantial.

Specific Utility

Applicants' asserted utility is "specific". "A 'specific utility' is specific to the subject matter claimed and can 'provide a well-defined and particular benefit to the public.'" *Id.* § 2107.01(I)(A) (quoting *Fisher*, 421 F.3d at 1371, 76 USPQ2d at 1230 (emphasis in original)). For the "specific" utility requirement, "an application must disclose a use which is not so vague as to be meaningless." *Fisher*, 421 F.3d at 1371, 76 USPQ2d at 1230. DGAT activity is specific because it is an enzymatic activity for the conversion of specific substrates, fatty acyl CoA and diacylglycerol, to specific products, triacylglycerols. Benefits to the public of DGAT activity include, for example, increasing the oil content of oilseeds when DGAT activity is overexpressed and diversion of carbon into other metabolites when DGAT activity is suppressed (page 1, lines 21-23). Further, DGAT activity cannot be called "vague",[2] as the nature of the reaction converting diacylglycerols to triacylglycerols (EC 2.3.1.20) has been known for quite some time. *See, e.g.*, Andersson *et al.*, J. Lipid. Res. 35:535-45 (1994) and Cases *et al.*, Proc. Natl. Acad. Sci. USA 95:13018-23 (1998), which each cite to a 28-year-old review article (Bell, Annu. Rev. Biochem. 49:459-87 (1980)) for background information on DGATs.

Credible Utility

Finally, Applicants' asserted utility is credible. "Credibility is assessed from the perspective of one of ordinary skill in the art in view of the disclosure and any other evidence of record (e.g., test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions." MPEP § 2107(II)(B)(1)(ii). "An applicant need only provide one credible assertion of

[2] According to Merriam-Webster's Dictionary, the definition of "vague" that appears to be most relevant here is "not clearly defined, grasped, or understood." *See* Merriam-Webster's Online Dictionary, at http://www.m-w.com/cgi-bin/dictionary?book=Dictionary&va=vague (last visited Oct. 7, 2007).

specific and substantial utility for each claimed invention to satisfy the utility requirement." *Id.*

The thrust of the Examiner's attack on the utility of the present invention appears to be directed at the credibility requirement. For example, the Examiner questions Applicants' assertion of DGAT activity based on sequence homology to other known DGATs and questions Applicants' 132 Declaration filed on May 17, 2007 (*hereinafter* "132 Declaration") as being based on a DGAT assay from a post-filing reference. August 8, 2007, Final Office Action, at 3 (*hereinafter* "Final Office Action"). The Examiner's questioning of Applicants' assertions and data is flawed, however, because while "an asserted use must show that that [sic] claimed invention has a significant and presently available benefit to the public," *Fisher*, 421 F.3d at 1371, 76 USPQ2d at 1230, an applicant can overcome a utility rejection "by suitable proofs indicating that the statement of utility and its scope as found in the specification are true." *Langer*, 503 F.2d at 1391-92, 183 USPQ at 297 (emphasis added). Evidence submitted to confirm a fact found in the specification as filed can include "after-filed" information. *See, e.g., In re Brana*, 51 F.3d 1560, 1567 n.19, 34 USPQ2d 1436, 1441 n.19 (Fed. Cir. 1995) (finding that a declaration "dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility)."). Here, the data described in the 132 Declaration was produced using a journal article published post-filing, but such data was submitted only to confirm a fact found in the specification as filed, namely, that the claimed invention has DGAT activity. Thus, the only review that the Examiner should have undertaken was whether the asserted DGAT activity was credible, that is, whether the "the reliability of the statement based on the logic and facts that are offered by the applicant [supported] the assertion of utility." MPEP § 2107.02(III)(B).

The 132 Declaration demonstrates that the asserted utility is credible. As described in the 132 Declaration, the soybean protein encoded by cDNA clone sr1.pk008.a8 and the wheat protein encoded by cDNA clone wr1.pk0119.b6:fis were expressed in the yeast *Saccharomyces cerevisiae*. The yeast strain used was a

mutant strain in which the DGAT gene, DGA1, and the PDAT gene, LRO1, had been deleted. Microsomal fractions were prepared from the transgenic yeast lines, and DGAT assays were performed using radioactively-labeled oleoyl-CoA and endogenous diacylglycerol. Triacylglycerol (TAG) was isolated by thin-layer chromatography and TAG was measured using liquid scintillation counting. The protein preparation from yeast transformed with the soybean gene produced 868 +/- 25 units of activity (pmol of TAG/min/mg of microsomal protein), the protein preparation from yeast transformed with the wheat gene produced 521 +/- 7 units of activity, and the protein preparation from yeast transformed with the vector control produced 21 +/- 10 units of activity. Consequently, the soybean protein encoded by sr1.pk008.a8 and the wheat protein encoded by cDNA clone wr1.pk0119.b6:fis were each shown to have DGAT activity.

Further, the showing of DGAT activity for only one species of the claimed genus is sufficient because the claimed sequences are related by 95% sequence identity. "Where an applicant has established utility for a species that falls within an identified genus of compounds, and presents a generic claim covering the genus, as a general matter, that claim should be treated as being sufficient under 35 U.S.C. 101." MPEP § 2107.02; *see also Brana*, 51 F.3d at 1567, 34 USPQ2d at 1442 ("[E]vidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility."). Here, the 132 Declaration demonstrates DGAT activity for one of the species within claim 26, a sequence having one amino acid difference from SEQ ID NO:16. 132 Declaration at 3-4. In accordance with MPEP § 2107.02, Applicants' data should be sufficient for the entire claimed genus.

From the facts of the present case, the only reasonable conclusion is that Applicants' statement of asserted utility is credible. Applicants' use of a test from a well-respected journal (The Journal of Biological Chemistry) and the conclusiveness of the results in the 132 Declaration should have convincingly established to the Examiner the credibility of the asserted utility. Further, office personnel

> must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office

> personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

MPEP § 2107(II). As noted above, the Examiner's countervailing evidence of lack of utility is weak at best, and is certainly not enough to tip the scale of preponderance of evidence towards lack of utility. Applicants should not have been required to describe the Andersson assay in the specification, because these experiments and other DGAT assays were well-known in the art at the time the present application was filed. Further, disregarding the 132 Declaration because it involved a test for DGAT activity developed post-filing was clear error, and, in any event, does nothing to refute the facts presented in the 132 Declaration, namely that SEQ ID NO:16 has DGAT activity as stated in the specification as filed.

In light of the above, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 101.

B. Claims 26 and 30-40 Comply with the Written Description Requirement of 35 U.S.C. § 112, 1st Paragraph.

1. Claims 26 and 32-40

Claim 26 is drawn to an isolated polynucleotide encoding a polypeptide having DGAT activity and at least 95% sequence identity to SEQ ID NO:16. Dependent claims 30-40 also require the isolated polynucleotide to encode a polypeptide having DGAT activity.

Applicants' claimed invention substantially conforms to Example 14 of the "Synopsis of Application of Written Description Guidelines", 66 Fed. Reg. 1099 (Jan. 5, 2001), *available at* http://www.uspto.gov/web/menu/written.pdf (last visited Oct. 5, 2007) (*hereinafter* "Written Description Guidelines"). In Example 14 of the Written Description Guidelines, the exemplary claim is directed to "A protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A→B." Written Description Guidelines, at 53. Included in the Example 14 specification are "indicat[ions] that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and . . . an assay for detecting the catalytic activity of the protein." *Id.* Under the "Analysis" section of

Example 14, the requirements of 95% identity to SEQ ID NO:3 and having catalytic activity "are essential to the operation of the claimed invention." *Id.* The procedures of making and testing sequences having 95% identity to SEQ ID NO:3 are determined to be "conventional." *Id.* Example 14 concludes that

> [t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are capable of the specified catalytic activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.

Id. at 54-55.

Applicants' claimed invention, though directed to the nucleotide sequences encoding the proteins having DGAT activity, is structured similarly to that of the Example 14 claim. The claimed nucleotide sequences encode proteins having 95% identity to SEQ ID NO:16, with the encoded proteins having DGAT activity. Like Example 14, there is not substantial variation in the encoded proteins, because the entire genus must have 95% sequence identity to SEQ ID NO:16 and have DGAT activity. Further, Applicants provided a DGAT assay, described above, used to identify the proteins having 95% sequence identity to SEQ ID NO:16 that also have DGAT activity. Procedures for producing proteins having 95% identity to SEQ ID NO:16 are well-known in the art (*see, e.g.*, page 12, line 16 – page 13, line 35).

Applicants also take issue with the Examiner's statement that a further reason for claim 26 failing the written description requirement was that "neither the specification nor the prior art discloses any polypeptide that is at least 90% identical to SEQ ID NO:16 except for SEQ ID NO:16 itself." Final Office Action, at 4. First, if the prior art disclosed a sequence having 90% identity to SEQ ID NO:16, then claim 26 in its version set forth in the Response to NF Office Action could possibly be obvious for claiming a nucleotide sequence of a known protein. A claim to a protein having 90% identity to SEQ ID NO:16, such as that in the canceled claims directed to amino acid sequences having 90% identity to SEQ ID NO:16, would be anticipated. As the Examiner appears to acknowledge through the absence of section 102 and 103 rejections in either the NF Office Action or the Final Office

Action, the claimed sequences at 90% identity to SEQ ID NO:16 are novel and nonobvious over the prior art, so disclosure of prior art sequences within the scope of claim 26 is not possible without anticipating the invention. Second, disclosing every variant of SEQ ID NO:16 having 90% identity thereto accomplishes nothing but creating a sequence listing of thousands of pages. The written description requirement is not so rigid. *Accord Falkner v. Inglis*, 448 F.3d 1357, 1367, 79 USPQ2d 1001, 1008 (Fed. Cir. 2006) ("[I]t is the binding precedent of [the Federal Circuit] that *Eli Lilly* does *not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art."). Having sufficient written description in a specification merely requires an application to show possession of the invention to one of ordinary skill in the art. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). By showing one sequence having 90% identity to SEQ ID NO:16 in the specification, that is SEQ ID NO:16 itself, the skilled artisan would know that every possible sequence having 90% identity thereto is readily ascertainable without reference to a sequence listing showing every possible variant of SEQ ID NO:16. The same of course is true for the even narrower claim of 95% identity to SEQ ID NO:16.

The lack of working examples and absence of disclosed structure should not affect the written description analysis. In *Falkner*, the Federal Circuit confirmed that

> (1) examples are not necessary to support the adequacy of a written description (2) [sic] the written description standard may be met . . . even where actual reduction to practice of an invention is absent; and (3) there is no *per se* rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

448 F.3d at 1366, 79 USPQ2d at 1007 (emphasis in original). *Falkner* also noted that "a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention." *Id.* at 1368, 79 USPQ2d at 1008. Possession of the claimed invention here relates to the

structure, identity to SEQ ID NO:16 (the structure of which is undisputed as evidenced by the actual sequence), correlated with function, DGAT activity.

Applicants thus respectfully request withdrawal of the rejection of claims 26 and 30-40 under 35 U.S.C. § 112, first paragraph, written description.

2. Claims 30 and 31

Claim 30 is directed to an isolated nucleic acid sequence comprising SEQ ID NO:15. The Examiner rejected claim 30 as failing to comply with the written description requirement, though neither the NF Office Action nor the Final Office Action specifically discusses this claim. Applicants thus assume that the reasons for rejecting claim 30 for failing to comply with the written description requirement are the same as those for claim 26.

Applicants do not understand how presentation of a defined sequence fails to show possession of a claimed invention. The USPTO routinely allows such claims, and the Written Description Guidelines specifically note in Example 8 that such claims accompanied by evidence of sequence homology to a known activity satisfy the written description requirement. Written Description Guidelines, at 33-35. In Example 8, a specific open reading frame ("ORF") is disclosed as having high homology to a DNA ligase, and the specification thus asserts that the ORF is a DNA ligase. *Id.* at 33. The sample claim in the Example reads "An isolated and purified nucleic acid comprising SEQ ID NO:2." *Id.* As Example 8 notes, this claim is drawn to a genus of nucleotide sequences that minimally contain the claimed sequence, and that a single species of this genus, the ORF itself, is disclosed. *Id.* at 34. Example 8 concludes that this disclosure is sufficient written description to support the sample claim. *Id.* at 35.

Here, Applicants claim a polynucleotide encoding an amino acid sequence that Applicants deduced to be a DGAT. Sequence homology to known DGATs evidences that SEQ ID NO:16 is a DGAT (see Example 8 of Applicants' Specification, at page 21, line 7 – page 23, line 4), which the 132 Declaration confirms. As such, Applicants believe that Example 8 of the Written Description Guidelines is directly on point with present claim 30 and, in light thereof, that the

Examiner should have found sufficient written description in Applicants' Specification for claim 30.

Turning to claim 31, it is broader than claim 30, but is still specifically defined by a sequence, that is a nucleotide sequence encoding SEQ ID NO:16. While Applicants only disclosed SEQ ID NO:15 as a nucleotide sequence encoding SEQ ID NO:16, because of the link between the genetic code and the amino acid sequences coded thereby (which is undeniably well known to the skilled artisan), all possible coding sequences for SEQ ID NO:16 can be readily determined by the skilled artisan without further assistance from the Applicants. Yet, as with claim 30, the Examiner rejected claim 31, without comment, as failing to comply with the written description requirement.

This situation is also covered by the Written Description Guidelines. In Example 11, the specification discloses a DNA sequence (SEQ ID NO:1) that encodes a cell surface receptor for adenovirus, designated protein X (SEQ ID NO:2). Written Description Guidelines, at 41. Sample claim 1 reads: "An isolated DNA that encodes protein X (SEQ ID NO:2)." *Id.* In the analysis of this claim, Example 11 notes that

> Claim 1 is drawn to a genus of DNAs that encode amino acid sequence SEQ ID NO:2, i.e., all sequences degenerately related by a genetic code table to SEQ ID NO:1. Although only one specie within the genus is disclosed, SEQ ID NO:1, a person of skill in the art could readily envision all the DNAs degenerate to SEQ ID NO:1 by using a genetic code table. One of skill in the art would conclude that applicant was in possession of the genus based on the specification and the general knowledge in the art concerning a genetic coding table.

Id. at 41-42. Applicants' claim 31 and Specification are no different. Claim 31 is drawn to a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:16. One species, SEQ ID NO:15, of the claimed genus is disclosed, but the skilled artisan can readily envision all other members of the genus because of the degeneracy of the genetic code. Thus, in accordance with Example 11 of the Written Description Guidelines, the Examiner should have determined that claim 31 is supported by sufficient written description.

Applicants thus respectfully request removal of the written description rejections for claims 30 and 31.

D. Because Claims 26 and 30-40 are Supported by Either a Specific and/or Substantial Asserted Utility or a Well Established Utility, Applicants have Enabled the Skilled Artisan to Use the Claimed Inventions.

Claims 26 and 30-40 were rejected under 35 U.S.C. §112, first paragraph, for lack of enablement, because the claimed invention is allegedly not supported by either a specific and/or substantial asserted utility or a well established utility. Applicants respectfully submit, for reasons cited in Section (VII)(A), *supra*, regarding the rejection under 35 U.S.C. § 101, that there is a specific and substantial utility for the claimed inventions. Applicants thus request removal of the related enablement rejections of claims 26 and 30-40.

E. Claims 26 and 30-40 are Enabled Under 35 U.S.C. § 112, 1st Paragraph.

Claims 26 and 30-40 were rejected for lack of enablement for sequences that encode a polypeptide with at least 95% sequence identity to SEQ ID NO:16, for lack of guidance as to which amino acid changes could be made and still produce a functional enzyme.

Applicants agree with the Examiner that a specification must enable one of ordinary skill in the art to make and use the claimed invention without undue experimentation. Applicants respectfully submit, however, that the Examiner's conclusion of nonenablement of sequences having 95% identity to SEQ ID NO:16 is erroneous because any experimentation needed to practice the present invention would be routine. "[A] patent specification complies with the statute even if a 'reasonable' amount of routine experimentation is required in order to practice a claimed invention, but that such experimentation must not be 'undue.'" *Enzo Biochem, Inc. v. Calgene, Inc.*, 118 F.3d 1362, 1371, 52 USPQ2d 1129, 1135 (Fed. Cir. 1999). Factors to consider when deciding whether experimentation is undue include: "(1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in

the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims." *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Applicants address each of the *Wands* factors below.

(1) The quantity of experimentation needed is quite low. As noted above, methods of producing nucleotide sequences are well-known in the art. The method of testing for DGAT activity cited in the specification is known in the art and has been relied on several times by other research groups for showing DGAT activity. For example, Cases *et al.* (Proc. Natl. Acad. Sci. USA 95:13018-23 (1998)) use a modification of the procedure of Andersson *et al.* to assay DGAT activity.

(2) The specification provides sufficient direction for producing nucleotide sequences encoding proteins having 95% identity to SEQ ID NO:16, and a specific assay for DGAT activity is provided.

(3) Applicants admit that there are no working examples showing DGAT activity in the specification. The specification's lack of working examples, however, does not automatically equate to nonenablement of the claimed invention.

> A claim will not be invalidated on section 112 grounds simply because the embodiments of the specification do not contain examples explicitly covering the full scope of the claim language. That is because the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before.

LizardTech, Inc. v. Earth Resource Mapping, Inc., 434 F.3d 1336, 1345, 76 USPQ2d 1724, 1732 (Fed. Cir. 2005) (internal citation omitted).

(4) The invention is one of nucleotide sequences encoding proteins having DGAT activity. Such an invention requires some experimentation for even routine techniques.

(5) Applicants' first provisional application, U.S. Patent Application Serial No. 60/110,602, filed December 2, 1998, contained partial sequences for a DGAT protein from corn (SEQ ID NO:4), rice (SEQ ID NO:12), soybean (SEQ ID NO:18) and wheat (SEQ ID NO:20). Applicants' second provisional application, U.S. Patent Application Serial No. 60/127,111, filed March 3, 1999, contained complete coding sequences for the DGAT protein from soybean (SEQ ID NO:16) and Arabidopsis (SEQ ID NO:2). Prior to filing of Applicants' first provisional application, the sequence was known for a mouse DGAT gene (Cases *et al.*, Proc. Natl. Acad. Sci. USA 95:13018-

23 (1998)) and a putative human DGAT gene (Oelkers *et al.*, J. Biol. Chem. 273:26765-71 (1998)). Additionally, a mutant in Arabidopsis, AS11, in the TAG1 locus was known that resulted in plants with reduced DGAT activity (Katavic *et al.*, Plant Physiol. 108:399-409 (1995)). The TAG1 locus was subsequently identified as encoding the DGAT gene (Zou *et al.*, Plant J. 19:645-53 (1999)). In determining the DGAT activity of the mouse gene, Cases *et al.* used DGAT coding sequences with or without an N-terminal FLAG epitope (MG**DYKDDDD**G-, epitope in bold font). The DGAT activity level increased proportionately with the amount of FLAG-tagged protein (page 13020; left column), indicating that the mouse gene encodes a DGAT protein and that modification of the N-terminus did not destroy the DGAT activity.

(6) This invention is related to the biotechnical arts in a well-known pathway, triacylglycerol synthesis, and the skill level of the artisan is very high. The skilled artisan is therefore very familiar with the pathway and well versed in many methods and techniques of, for example, gene manipulation, protein synthesis, and enzyme action.

(7) Claim 26 is directed to a nucleotide sequence encoding a protein having a specified activity. It is unreasonable for Applicants to provide a cookbook recipe of how to practice the invention. Rather, Applicants have depended on the skill and experience of the skilled artisan to implement the invention using nucleotide sequences encoding enzymes having DGAT activity. Applicants expect that the skilled artisan would be aware of successful molecular biology and biochemistry methods and therefore be capable of producing the described sequences and testing these sequences for DGAT activity.

(8) The Examiner's concerns about the number of possible sequences having 95% identity to SEQ ID NO:16 are unfounded. Indeed, the number of possible claimed sequences should not itself form the basis of an enablement rejection. *See, e.g., Novozymes A/S v. Genencor Int'l, Inc.*, 446 F. Supp. 2d 297, 330 (D. Del. 2006) (noting that, with claims covering polypeptides having 95% identity to a disclosed sequence, a "large number [of possible sequences] alone is not sufficient to show a lack of enablement . . .").

Outside of factor (3), the *Wands* factors support Applicants assertion that any experimentation required to practice the present claims would be routine. "It is well

established that a patent applicant is entitled to claim his invention generically when he describes it sufficiently to meet the [enablement requirement]." *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991). In *Amgen*, the court found that a generic claim covering all possible DNA sequences encoding any polypeptide having an amino acid sequence "sufficiently duplicative" of erythropoietin ("EPO") and which causes bone marrow cells to increase production of reticulocytes and red blood cells, and increases hemoglobin synthesis or iron uptake as being nonenabled where the patentee only provided information "of how to make EPO and very few analogs." *Id.* at 1213-14, 18 USPQ2d at 1027. As noted in *Novozymes*, however, "[t]he problem in *Amgen* was that the claim scope covered <u>any</u> gene that could be used to express proteins of various sizes that had one or more of the biological properties of EPO." *Novozymes*, 446 F. Supp. 2d at 330 (emphasis added). Unlike the patentee in *Amgen*, Applicants are not claiming <u>all</u> nucleotide sequences encoding enzymes having DGAT activity but merely those having 95% identity to SEQ ID NO:16. Even the *Amgen* court recognized that the enablement requirement should not be extended beyond reasonableness when it noted that the disclosure at issue there might have been sufficient to enable a claim for EPO analogs similar to those described in that specification. *Amgen*, 927 F.2d at 1213, 18 USPQ2d at 1027 (noting that the patentee's "disclosure might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for [patentee's] desire to claim all EPO gene analogs").

Applicants' situation is similar to that in *Novozymes*. There, patentee's claim 1 read:

> A variant of a parent *Bacillus stearothermophilus* alpha-amylase, wherein the variant has an amino acid sequence which has at least 95% homology to the parent *Bacillus stearothermophilus* alpha-amylase and comprises a deletion of amino acids 179 an [sic] 180, using SEQ ID NO:3 for numbering, and wherein the variant has alpha-amylase activity.

Novozymes, 446 F. Supp. 2d at 306. The *Novozymes* court concluded that "requiring at least 95% homology with [the identified sequence] makes the variants sufficiently similar so that the enablement requirement is satisfied. By contrast to *Amgen*, the claim scope [in *Novozymes*] is limited quantitatively to similarity between protein sequences and not just to a requirement for alpha-amylase-like activity."

Novozymes, 446 F. Supp. 2d at 300. Applicants' current claims are similarly structured. There is a quantitative limit to the similarity between SEQ ID NO:16 and other proteins in the claimed genus and all proteins having 95% identity to SEQ ID NO:16 must have DGAT activity. Thus, Applicants' claimed invention should be sufficiently enabled.

Ex parte Kubin, 83 USPQ2d 1410 (BPAI 2007), also supports Applicants' enablement arguments. The *Kubin* Appellants claimed polynucleotides encoding polypeptides having 80% identity to a defined amino acid sequence, which has a defined binding activity. *Kubin*, 83 USPQ2d at 1412. The Board found that Appellants' specification taught how to make variants of the defined amino acid sequence, how to calculate identity between the defined amino acid sequence and the variants, and how to test the variant for the claimed binding activity. *Id.* at 1416. The specification did not disclose, however, which amino acids could be changed and still retain the claimed activity, and it did not disclose any actual variants of the defined amino acid sequence. *Id.* at 1415-16. The examiner in *Kubin* rejected the claims as lacking enablement for sequences having identity to the defined amino acid sequence because of the absence of working examples and because changes in defined amino acid sequence might alter the function of the variant as compared to the defined amino acid sequence. *Id.* at 1415. The examiner there also noted the unpredictability of the molecular biology art. *Id.* at 1416. In finding enablement of the claimed invention, the Board agreed with the examiner that the molecular biology art was unpredictable (*Wands* factor 7), but "the other *Wands* factors weigh[ed] in Appellants' favor, particularly the state of the art and the relative skill of those in the art as evidenced by the prior art teachings and Appellants' Specification." *Id.* at 1416 (internal citations and markings omitted). Further, the Board noted that "[t]he amount of experimentation to practice the full scope of the claimed invention might have been extensive, but it would have been routine. The techniques necessary to do so were well known to those skilled in the art." *Id.* (emphasis added). Like the *Kubin* Appellants, Applicants here provided teachings on how to make variants of SEQ ID NO:16 (*see, e.g.,* Applicants' Specification at page 12, line 20 – page 14, line 4), described how to calculate the sequence identities between SEQ ID NO:16 and its variants (*see, e.g.,* Applicants' Specification at page 7, line 34 – page 8, line 16), and

provided the Andersson assay to test for DGAT activity. Thus, *Kubin* dictates that Applicants' claims are enabled.

Further evidencing enablement of claim 26 is that the novel aspect of the invention is enabled in the specification. In a recent Federal Circuit case, the court clarified that "[a]lthough the knowledge of one skilled in the art is indeed relevant [to an enablement determination], the novel aspect of an invention must be enabled in the patent." *Auto. Techs. Int'l, Inc. v. BMW of N. Am., Inc.*, 501 F.3d 1274, 1283, 84 USPQ2d 1108, 1114-15 (Fed. Cir. 2007). In the present application, the novel aspect of the invention is the sequence set forth in SEQ ID NO:16 and variants thereof. As SEQ ID NO:16 was present in the sequence listing, which is considered part of the specification as filed, the novel aspect of the invention is enabled in the specification. Whether or not the claimed sequences have DGAT activity is irrelevant to the novelty of the claimed sequences; a claim directed solely to "a nucleotide sequence encoding an amino acid sequence having 95% identity to SEQ ID NO:16" would be novel without the DGAT activity limitation, which is present for section 112 purposes only. Indeed, DGAT activity itself is not novel; as the specification notes, "[a]cyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) uses fatty acyl CoA and diacylglycerol as substrates to catalyze the only committed step in triacylglycerol synthesis" (page 1, lines 13-15). Therefore, this knowledge can be imputed from those skilled in the art to supplement the present disclosure, as routine experimentation (a DGAT assay) provides the determination of whether a sequence having 95% identity to SEQ ID NO:16 is within the scope of the claim 26 invention. *See Invitrogen Corp. v. Clontech Labs., Inc.*, 429 F.3d 1052, 1070-71, 77 USPQ2d 1161, 1173 (Fed. Cir. 2005) ("The scope of enablement . . . is that which is disclosed in the specification plus the scope of what would be known to one of ordinary skill in the art without undue experimentation.").

Applicants further note that, should the office here limit Applicants' claimed invention to only those nucleotide sequences encoding SEQ ID NO:16, Applicants' patent rights become essentially useless because the skilled artisan could simply modify one amino acid of SEQ ID NO:16 (the sequence of which is undisputedly disclosed in the specification), confirm DGAT activity by the Andersson assay (undisputedly referenced in the specification), yet be outside the scope of the

Applicants' claims even though Applicants' specification disclosed the complete roadmap to working around the exceptionally narrow claims. In essence, the Examiner's scope of enablement rejection produces the absurd result of the specification enabling the skilled artisan to avoid infringement of claims covering only nucleotide sequences encoding SEQ ID NO:16, but the same specification failing to enable the same skilled artisan to produce the same modified amino acid sequence if the claims cover sequences having 95% identity to SEQ ID NO:16.

Applicants also believe that any of the arguments presented in the enablement section should be applicable towards establishing that sufficient written description was present in the specification as filed and vice versa. As noted in *LizardTech*, "a recitation of how to make and use the invention across the full breadth of the claim is ordinarily sufficient to demonstrate that the inventor possesses the full scope of the invention, and vice versa." 434 F.3d at 1345, 76 USPQ2d at 1732. That the present specification supports possession (written description) of the genus of polypeptides encompassed by the present claims (see above) further evidences enablement of the present claims. All methods for generating the described polypeptide variants were standard in the art at the time of filing. Likewise, methods for testing for the required activity were described in the specification (see above). Thus, the possessed genus is enabled, almost by definition.

In view of the foregoing, Applicants respectfully request withdrawal of the Section 112, 1st paragraph, enablement rejections of claims 26 and 30-40.

VIII. CONCLUSION

For the reasons set forth above, Applicants respectfully request that the Board reverse the final rejection of pending Claims 26 and 30-40 and indicate allowability of all claims.

Please charge any fee due which is not accounted for to Deposit Account No. 04-1928 (E.I. du Pont de Nemours and Company).

By: _____
Jeffrey Safran
Attorney for Applicants
Reg. No.: 54,689
Telephone: (302) 984-6132
Facsimile: (302) 658-1192

Dated: July 8, 2008

CLAIMS APPENDIX

26. An isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having diacylglycerol acyltransferase activity, wherein the amino acid sequence of the polypeptide and the amino acid sequence of SEQ ID NO:16 have at least 95% sequence identity, based on the Clustal alignment method with pairwise alignment default parameters of KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5, or

(b) the full-length complement of the nucleotide sequence of (a).

30. The polynucleotide of claim 26, wherein the nucleotide sequence comprises the nucleotide sequence of SEQ ID NO:15.

31. The polynucleotide of claim 26, wherein the amino acid sequence of the polypeptide comprises the amino acid sequence of SEQ ID NO:16.

32. A vector comprising the polynucleotide of claim 26.

33. A recombinant DNA construct comprising the polynucleotide of claim 26 operably linked to at least one regulatory sequence.

34. A method for transforming a cell comprising transforming a cell with the polynucleotide of claim 26.

35. A cell comprising the recombinant DNA construct of claim 33, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell and a plant cell.

36. A virus comprising the recombinant DNA construct of claim 33.

37. A method for producing a transgenic plant comprising transforming a plant cell with the polynucleotide of claim 26 and regenerating a transgenic plant from the transformed plant cell.

38. A plant comprising the recombinant DNA construct of claim 33.

39. A seed comprising the recombinant DNA construct of claim 33.

40. A method for isolating a polypeptide encoded by the recombinant DNA construct of claim 33 comprising:

(a) transforming a cell with the recombinant DNA construct of Claim 33;

(b) growing the transformed cell of step (a) under conditions suitable for expression of the recombinant DNA construct; and

(c) isolating the polypeptide from the transformed cell of step (b).

EVIDENCE APPENDIX

Attached herewith is a Declaration under 37 C.F.R. § 1.132 signed by Dr. Keith R. Roesler, which was filed on May 17, 2007, along with the Response to NF Office Action. The Examiner acknowledged submission of the 132 Declaration in the Final Office Action.

Also attached are the following journal articles (all admitted into the record by the Examiner on November 2, 2006):

Andersson *et al.*, J. Lipid. Res. 35:535-45 (1994)

Cases *et al.*, Proc. Natl. Acad. Sci. USA 95:13018-23 (1998)

Oelkers *et al.*, J. Biol. Chem. 273:26765-71 (1998)

Katavic *et al.*, Plant Physiol. 108:399-409 (1995)

Zou *et al.*, Plant J. 19:645-53 (1999)

-PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

CAHOON ET AL. CASE NO: BB1295 US CNT

SERIAL NO: 10/690,994 GROUP ART UNIT: 1638

FILED: OCTOBER 21, 2003 EXAMINER: L. ZHENG

FOR: PLANT DIACYLGLYCEROL ACYLTRANSFERASES

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Keith R. Roesler, declare that:

I am a citizen of the United States and reside at 3928 Patricia Drive, Urbandale, Iowa, 50322.

I am an employee of Pioneer Hi-Bred International, Inc., ("Pioneer") a subsidiary of E.I. du Pont de Nemours and Company ("DuPont").

I received a Ph.D. in Agronomy from the University of Illinois at Urbana-Champaign. I have worked for Pioneer from July 1, 1995, to the present in the fields of biochemistry and molecular biology.

I am familiar with the subject matter of the above-identified application and supervised the performance of the experiments explained below.

The following are my remarks:

1. In the December 18, 2006, Non-Final Office Action related to the above-identified application, the Examiner *inter alia* rejected claims 26-40 under 35 U.S.C. § 101 because the claimed invention is allegedly not supported by either a specific and/or substantial asserted utility or a well-established utility.

2. Described herein are a series of experiments that establish a specific and substantial asserted utility for the claimed inventions.

3. The basic experimental procedure is as follows. A *Saccharomyces cerevisiae* strain was created with two genes deleted: the *DGA1* gene, encoding acyl-CoA:diacylglycerol acyltransferase (DGAT), and the *LRO1* gene, encoding phospholipid:diacylglycerol acyltransferase (PDAT). In this yeast strain, DGAT genes were overexpressed using a strong constitutive promoter from the yeast phosphoglycerate kinase gene, and using uracil selection. DGAT assays were done using microsomal membrane preps.

4. The method of Milcamps *et al.*, 2005, "Isolation of a gene encoding a 1,2-diacylglycerol-*sn*-acetyl-CoA acetyltransferase from developing seeds of *Euonymus alatus*", J. Biol. Chem. 280:5370-5377 (attached herewith), was followed, with minor changes. *Saccharomyces cerevisiae* cultures were grown to early stationary phase in 100 ml of SC media minus uracil. Following harvest, the yeast pellets were resuspended in 4 ml of 20 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 0.3 M $(NH_4)_2SO_4$. Two ml of glass beads were added, and cells were lysed by vortexing for 5 min. The lysate was centrifuged for 15 min at 1500 g at 6 °C. The supernatant was then centrifuged at 100,000 g for 1.5 hr at 6 °C. The microsomal pellet was resuspended in 500 µl of 100 mM potassium phosphate, pH 7.2, containing 10% glycerol and frozen in liquid nitrogen prior to storage at -80 °C. Protein concentrations were determined by the method of Bradford, using the Coomassie Plus reagent (Pierce), with bovine serum albumin as standard.

5. DGAT assays were done for 1 min at 25 °C with 50 mM potassium phosphate pH 7.2, 10 µM 1-^{14}C-labeled oleoyl-coenzyme A (50 mCi/mmol, Perkin Elmer), and 20 µg of microsomal protein, using endogenous diacylglycerol, in a total reaction volume of 100 µl. The reaction was started by addition of the microsomal membranes to the remainder of the reaction components. The assay was stopped and lipids were extracted with 2 ml of hexane:isopropanol (3:2) (Hara and Radin, 1978, "Lipid extraction of tissues with a low-toxicity solvent", Anal. Biochem. 90:420-426) containing 4 µl of unlabeled triacylglycerol (triolein, Sigma). Following vortexing for 10 sec, the phases were separated with 1 ml of 500 mM sodium sulfate and vortexing was again done for 10 sec. After 10 min, the upper phase was transferred to another tube and dried with nitrogen gas. The lipid was resolubilized in a small volume of hexane (approximately 100 to

150 µl) and applied to K6 silica TLC plates, which were developed in 80:20:1 (v/v/v) hexane:diethylether:acetic acid. Triacylglycerol was visualized and marked by staining in iodine vapor. After the stain faded, the triacylglycerol was scraped, and radioactivity was determined by liquid scintillation counting. The following results were obtained.

Construct	DGAT Activity (pmol ^{14}C-labeled TAG produced/min/mg microsomal protein)			
	Rep1	Rep2	Rep3	Mean ± SD
Soybean DGAT	840	887	876	868 ± 25
Wheat DGAT	518	529	515	521 ± 7
Vector control	33	14	17	21 ± 10

6. The soybean DGAT protein-coding region was obtained by PCR using clone sr1.pk0098.a8 as a template. This sequence was used to create the yeast expression vector, PHP32069 (Appendix A). The sequence of the soybean DGAT-coding region, "PHP32069 Soy DGAT1 CDS", was found to have a one nucleotide difference with the corresponding DGAT-coding region of SEQ ID NO:15, "BB1295 SEQ-15 CDS". An alignment of the two DGAT-coding sequences is presented in Appendices B1-B4. The soybean DGAT sequence in PHP32069 has an adenine (A) at position 939 of the DGAT-coding region, while the corresponding nucleotide in SEQ ID NO:15 is cytosine (C). The cDNA insert in clone sr1.pk0098.a8 was re-sequenced, and the sequence of the DGAT-coding region in sr1.pk0098.a8 was identical to the sequence of the PCR-derived DGAT-coding region in PHP32069. The single nucleotide difference in SEQ ID NO:15, also obtained by sequencing clone sr1.pk0098.a8, is presumably due to a sequencing error. This single nucleotide difference results in a one amino acid difference between the two corresponding amino acid sequences (Appendix C). The DGAT protein encoded by PHP32069 has a glutamic acid residue (E) at position 313, while the corresponding residue in SEQ ID NO:16 is aspartic acid (D).

7. The wheat DGAT DNA was obtained by a combination of PCR using clone wr1.pk0119.b6:fis as a template and a synthetic gene fragment to complete the coding region. This DGAT-coding sequence was used to create the yeast expression vector, PHP32068 (Appendix D). The sequence of this wheat DGAT-

coding region, "PHP32068 Wheat DGAT1-2 CDS", was found to have two single nucleotide differences with the corresponding DGAT-coding region of SEQ ID NO:21, "BB1295 SEQ-21 CDS". An alignment of the two DGAT-coding sequences is presented in Appendices E1-E4. The wheat DGAT nucleotide sequence of PHP32068 has a guanosine (G) at position 303 and a thymidine (T) at position 393; the sequence of SEQ ID NO:21 has a thymidine (T) and cytosine (C) at these two positions, respectively. These two nucleotide differences are "silent", i.e., the amino acid sequence encoded by PHP32068 is identical to that of SEQ ID NO:22 (Appendix F).

8. Sequence alignments were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal V method of alignment with the default parameters

9. The vector control contained no DGAT gene.

10. As shown in the table above, both the soybean DGAT protein encoded by PHP32069 and the wheat DGAT protein encoded by PHP32068 have significant DGAT activity.

11. I believe that the experiments conducted thus establish a specific and substantial utility for the claimed inventions.

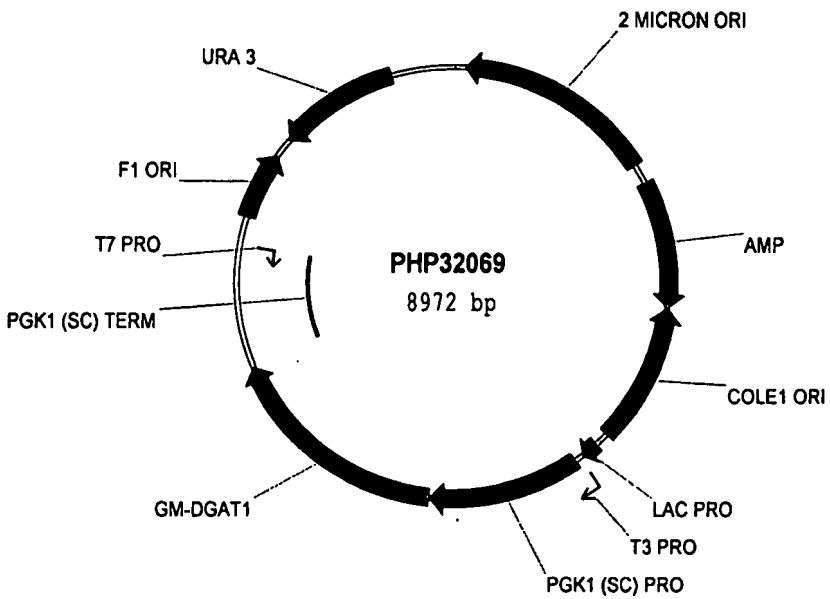
12. I declare that all statements made herein are either based on my own knowledge and are true, or if based on information and belief are believed to be true. I also declare that all statements were made with knowledge that willful false statements, and the like, are punishable by either fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and any such willful false statements may jeopardize the validity of either the patent application, or any patent issuing thereon.

By: Keith R. Roesler

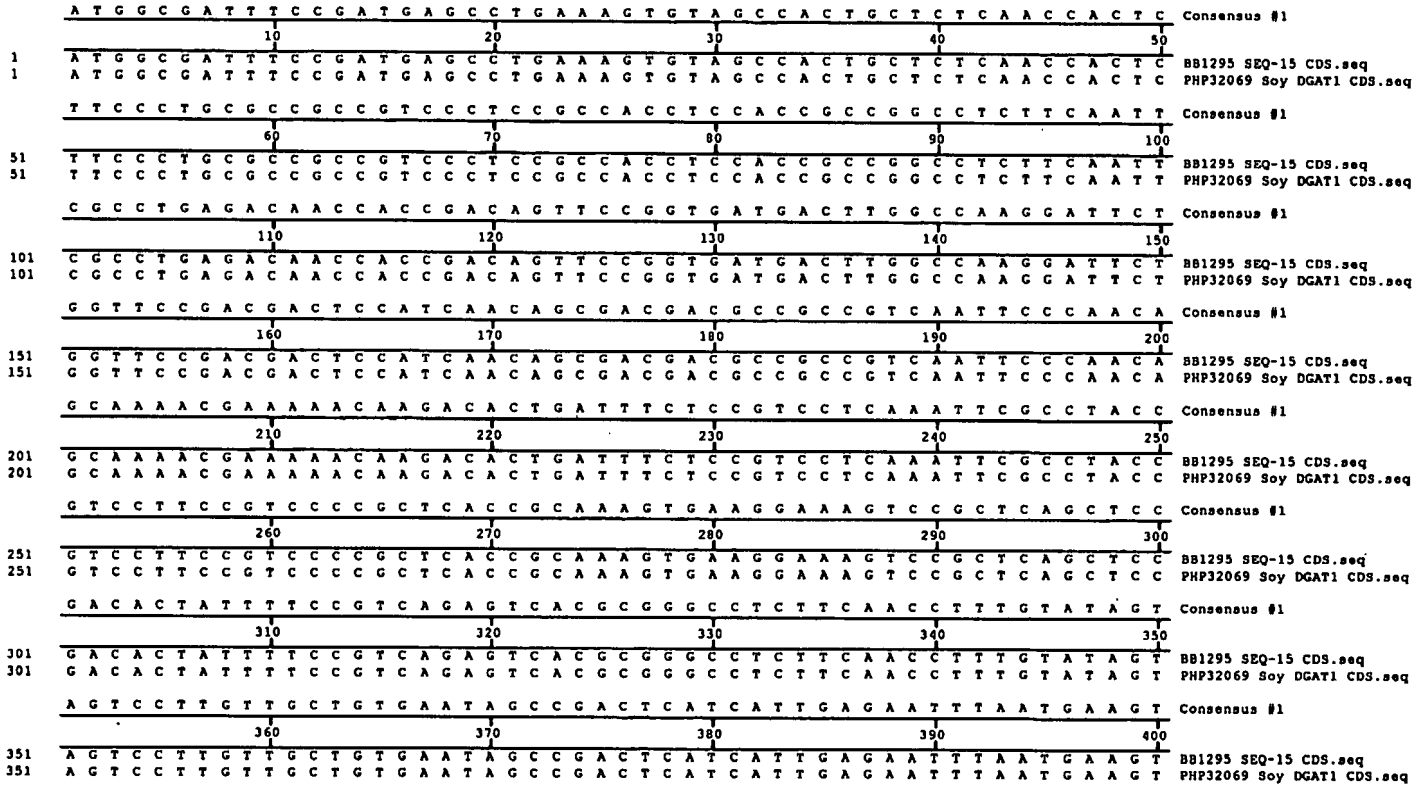
Keith R. Roesler

Dated: 5-14-07

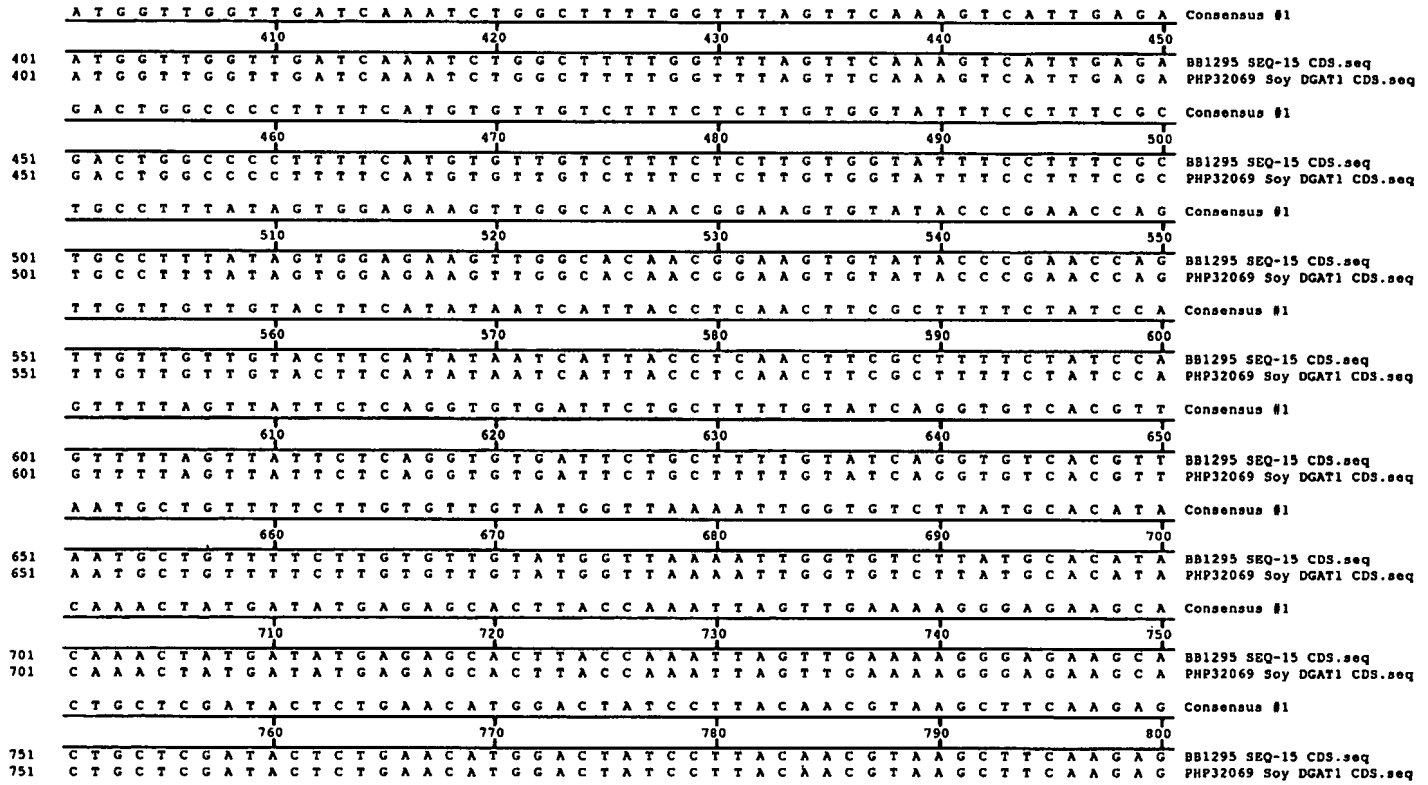
APPENDIX A
YEAST EXPRESSION VECTOR FOR SOYBEAN DGAT



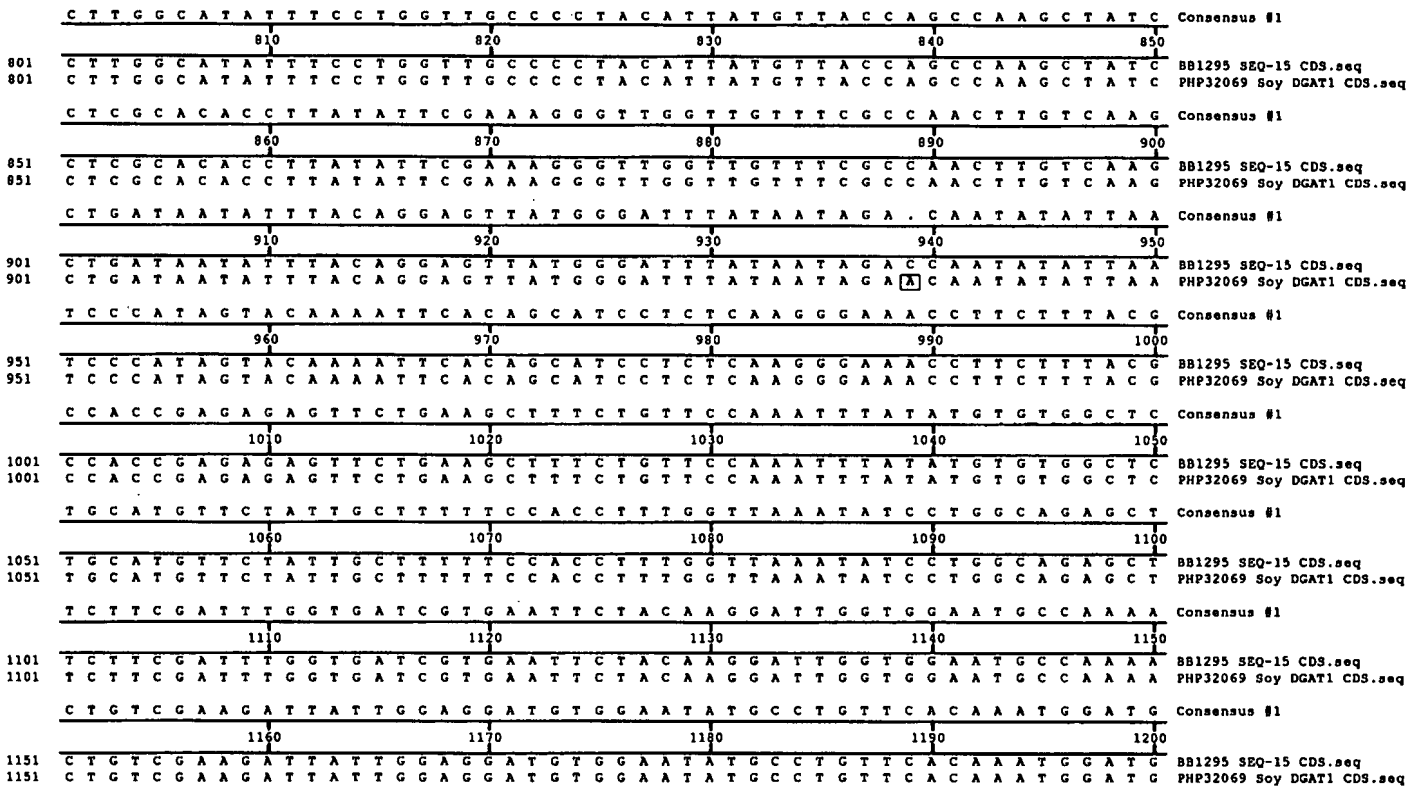
APPENDIX B1
SOYBEAN DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



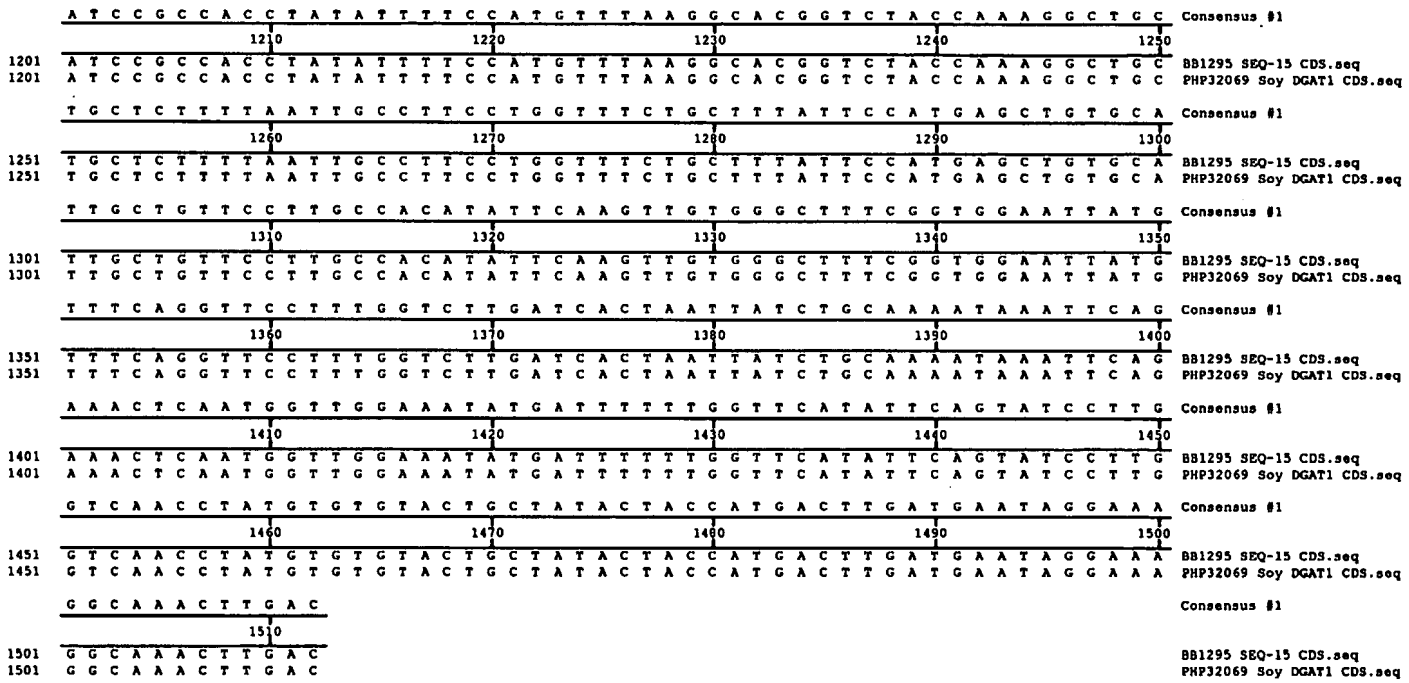
APPENDIX B2
SOYBEAN DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



APPENDIX B3
SOYBEAN DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



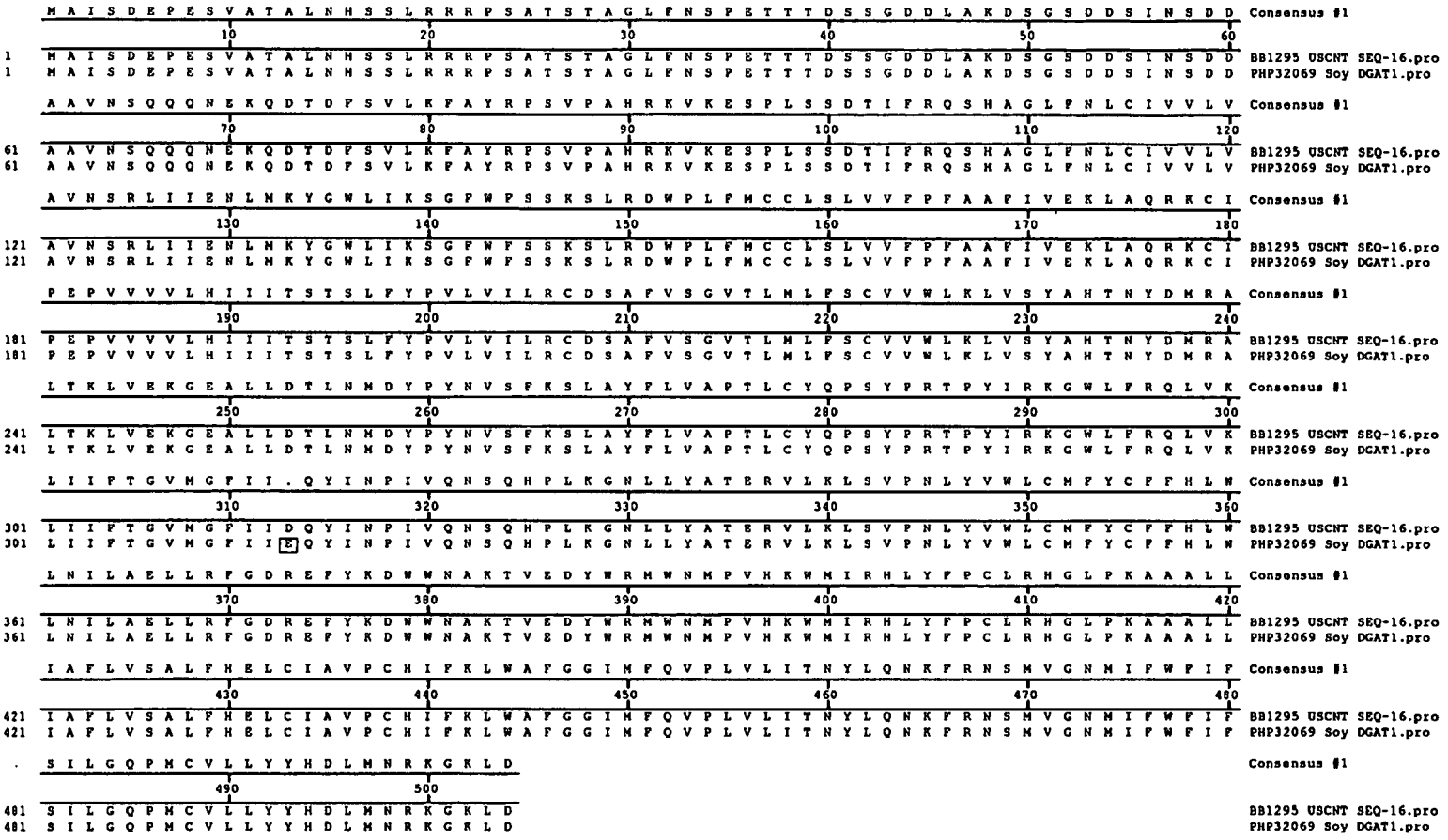
APPENDIX B4
SOYBEAN DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



Consensus 'Consensus #1': When all match the residue of BB1295 SEQ-15 CDS.seq show the residue of BB1295 SEQ-15 CDS.seq, otherwise show '.'.□

Decoration 'Decoration #1': Box residues that differ from BB1295 SEQ-15 CDS.seq.

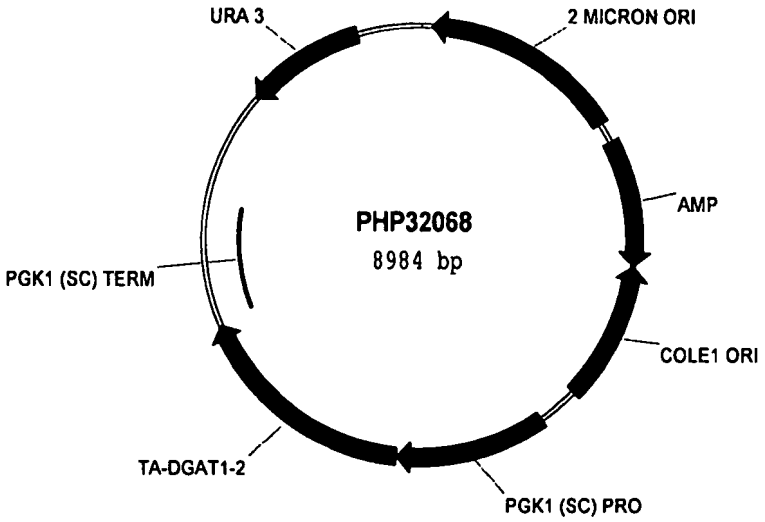
APPENDIX C
SOYBEAN DGAT AMINO ACID SEQUENCE ALIGNMENT



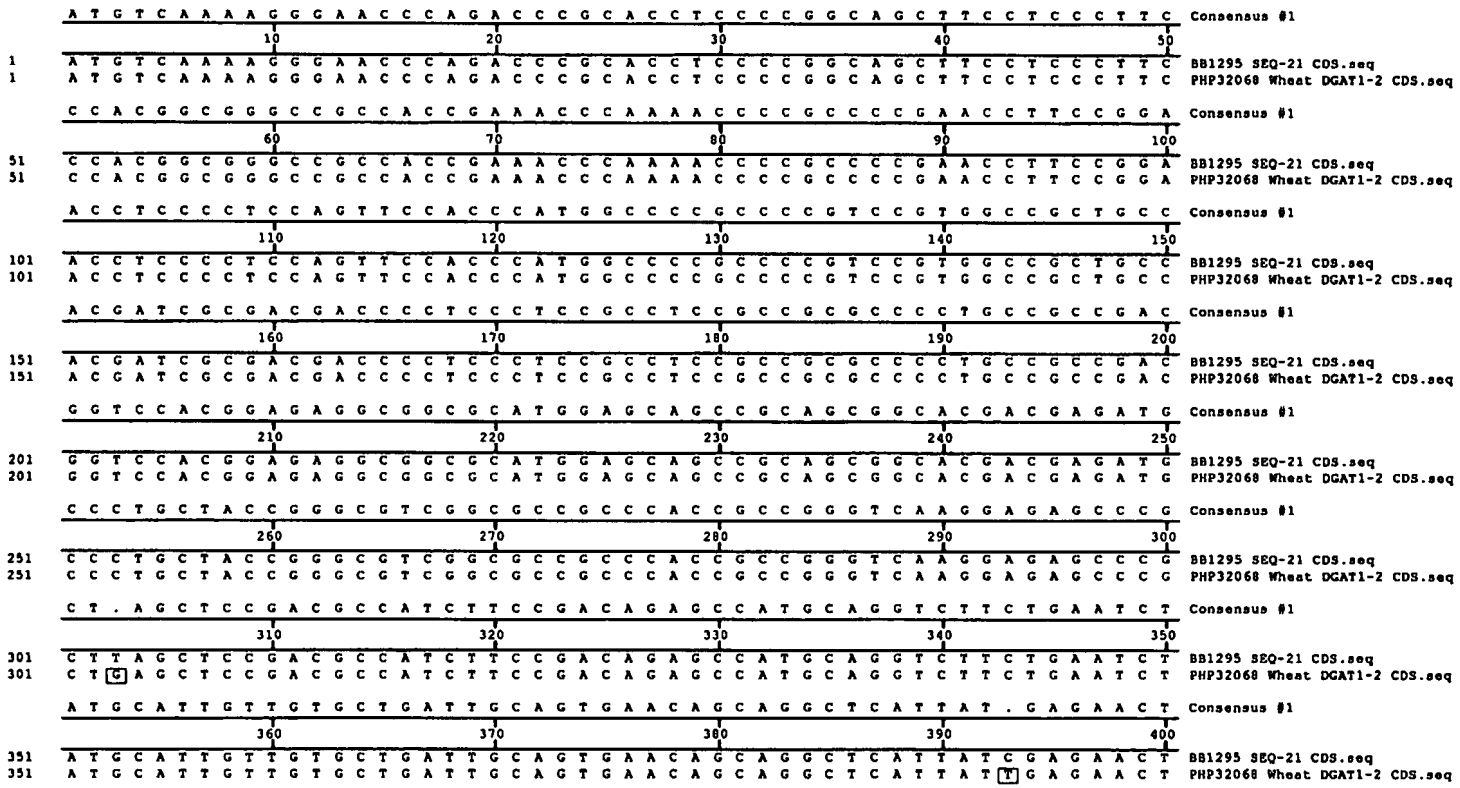
Consensus 'Consensus #1': When all match the residue of BB1295 USCNT SEQ-16.pro show the residue of BB1295 USCNT SEQ-16.pro, otherwise show '.'.

Decoration 'Decoration #1': Box residues that differ from BB1295 USCNT SEQ-16.pro.

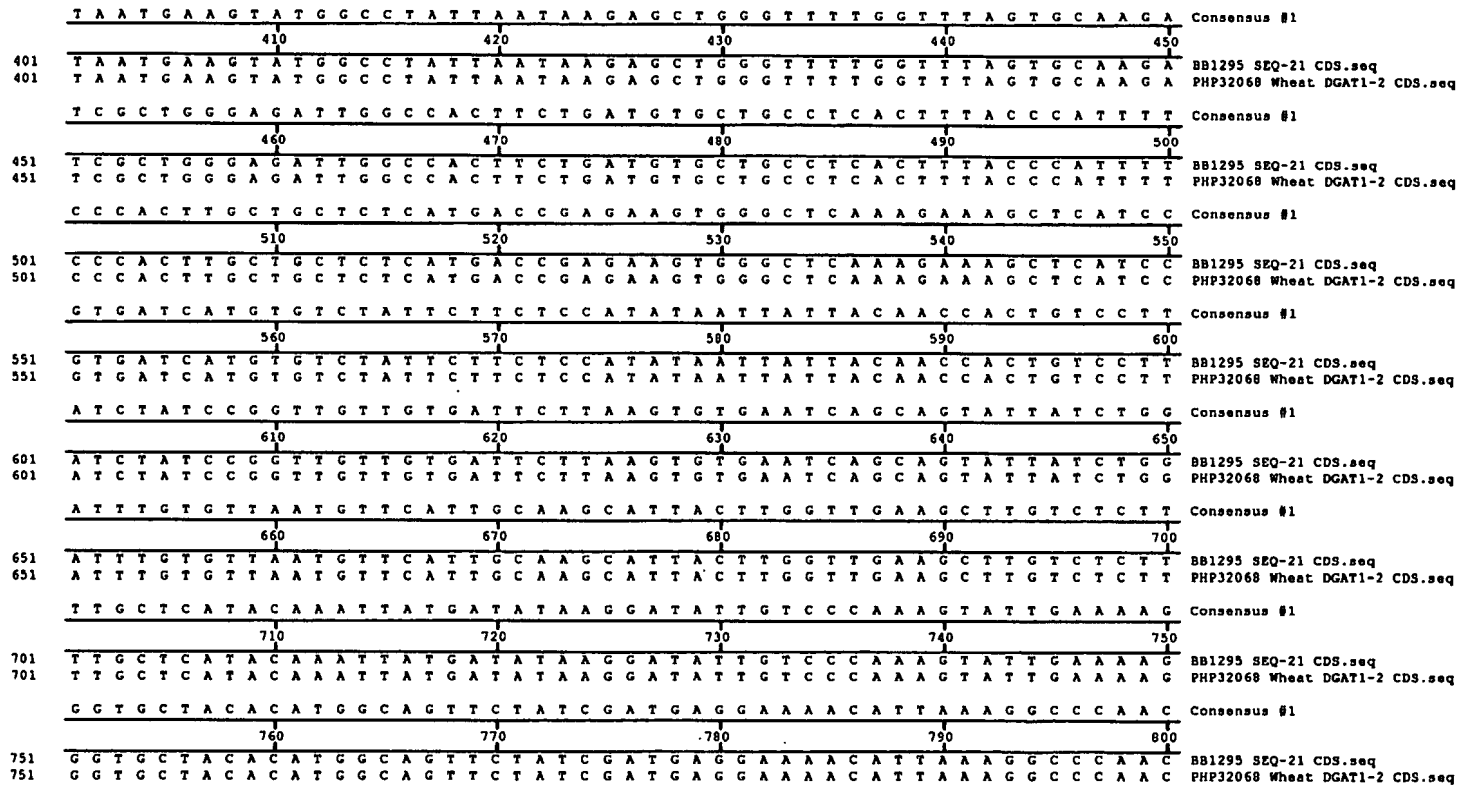
APPENDIX D
YEAST EXPRESSION VECTOR FOR WHEAT DGAT



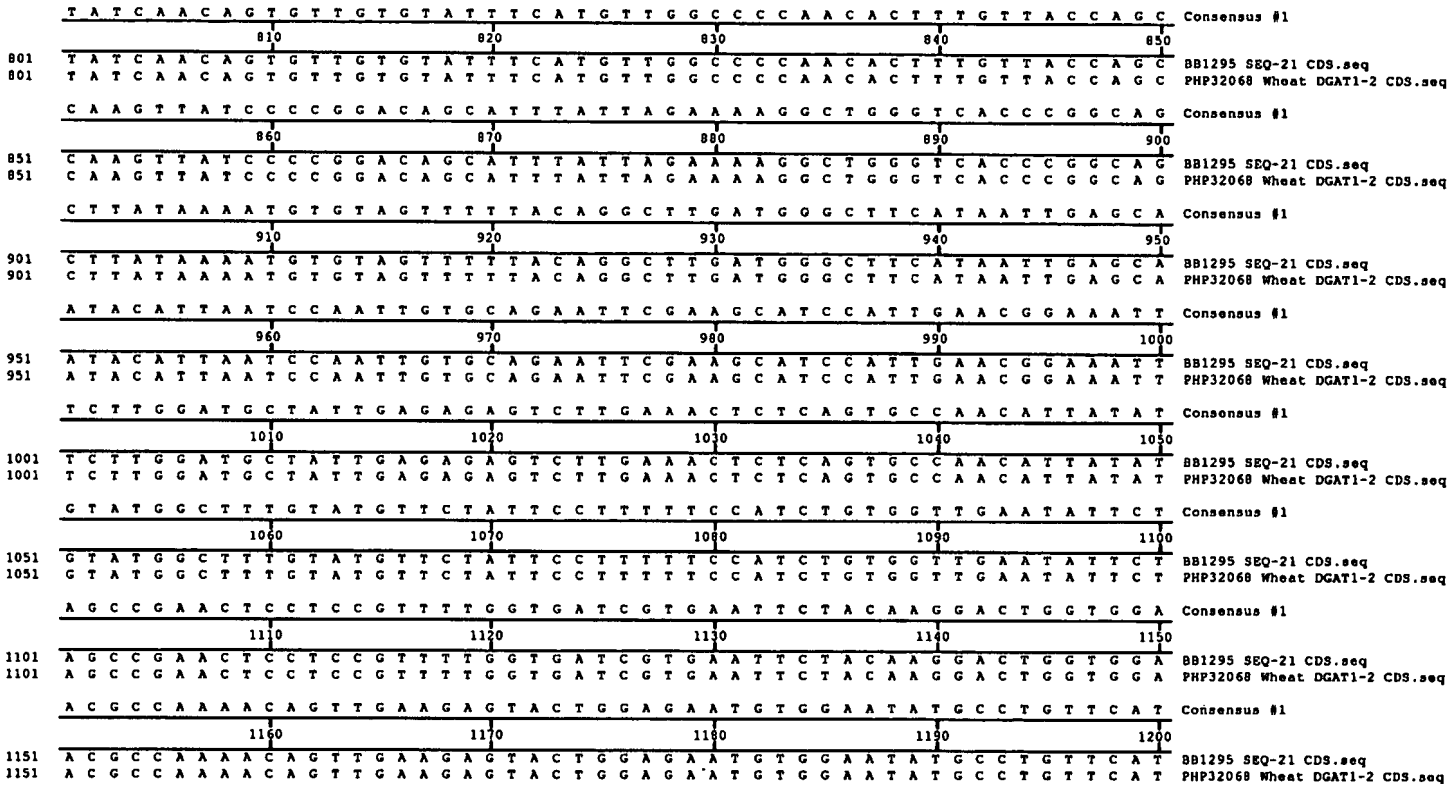
APPENDIX E1
WHEAT DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



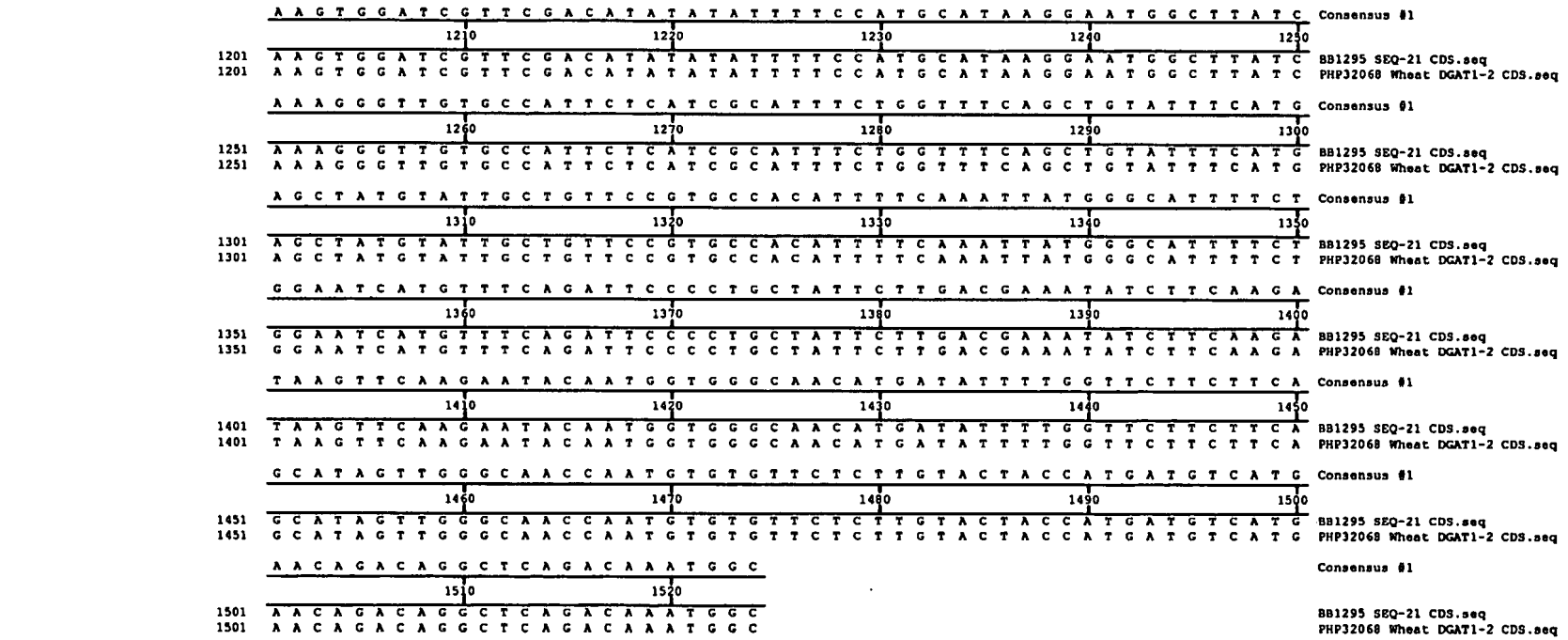
APPENDIX E2
WHEAT DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



APPENDIX E3
WHEAT DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



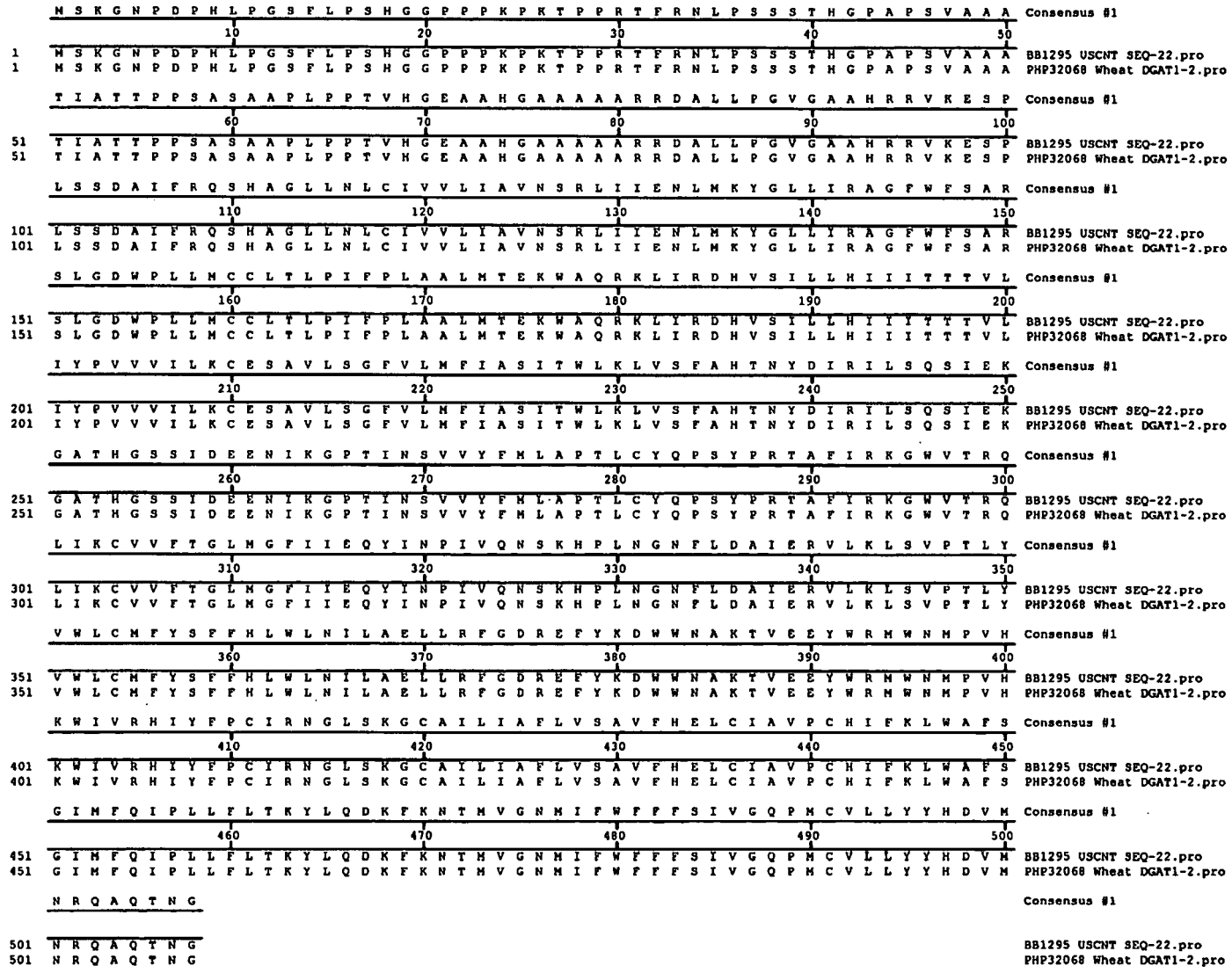
APPENDIX E4
WHEAT DGAT NUCLEOTIDE SEQUENCE ALIGNMENT



Consensus 'Consensus #1': When all match the residue of BB1295 SEQ-21 CDS.seq show the residue of BB1295 SEQ-21 CDS.seq, otherwise show '.'.□

Decoration 'Decoration #1': Box residues that differ from BB1295 SEQ-21 CDS.seq.

APPENDIX F – WHEAT DGAT AMINO ACID SEQUENCE ALIGNMENT



Consensus 'Consensus #1': When all match the residue of BB1295 USCNT SEQ-22.pro show the residue of BB1295 USCNT SEQ-22.pro, otherwise show '.'.□

Purification of diacylglycerol:acyltransferase from rat liver to near homogeneity

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Abstract A method to isolate a protein related to the diacylglycerol:acyltransferase (DGAT) activity in rat liver microsomes has been developed. The microsomes were treated with sodium deoxycholate (DOC; 0.1 mg/mg protein) at a concentration of 1 mM, i.e., below the critical micellar concentration (CMC), to remove luminal and loosely bound proteins. Three percent of the DGAT activity and all of the acylCoA hydrolyse activity were present in the supernatant, i.e., among the extracted loosely bound proteins. The insoluble material, recovered as a pellet, was suspended in DOC (1.6 mg/ml and mg protein in the original microsomes), and subjected to multiple, short (1-2 sec) sonications. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 5 mg/ml) was then added, and the sonication was repeated. The detergent-treated microsomal membranes were filtered through a 0.22-μm filter and chromatographed on a Superose 6 column from which the DGAT activity was recovered in a high molecular mass fraction. A monoclonal antibody that reacted with this fraction was raised and used in immunoaffinity experiments. This antibody removed 93 ± 6% (mean ± SD, n = 4) of the DGAT activity present in solution and 44 ± 6% (mean ± SD, n = 5) of the applied activity could be recovered after desorption. The antibody recognized a 60 kDa protein upon Western blot of rat liver microsomal proteins as well as of the DGAT-containing fraction from the Superose 6 column. A 60 kDa protein was highly enriched in the DGAT-containing retained fraction from the immunoaffinity chromatography. This 60 kDa protein reacted with the monoclonal antibody on Western blot. In addition to the 60 kDa protein, the retained fraction from the immunoadsorber contained a 77 kDa protein. This protein did not react with the monoclonal antibody on Western blots. Neither the 60 nor the 77 kDa protein reacted with antibodies to mouse immunoglobulins or showed any unspecific reaction with immunoglobulins.—**Andersson, M., M. Wettesten, J. Borén, A. Magnusson, A. Sjöberg, S. Rustaeus, and S-O. Olofsson.** Purification of diacylglycerol: acyltransferase from rat liver to near homogeneity. *J. Lipid Res.* 1994. 35: 535-545.

Supplementary key words diacylglycerol:acyltransferase (EC 2.3.1.20) • rat liver microsomes • detergent solubilization • monoclonal antibody • triglyceride biosynthesis

The synthesis of triacylglycerol has been suggested to occur in the endoplasmic reticulum (ER) and most of the enzyme activities involved appears to be bound to or associated with the membrane of this organelle (1). The majority of the steps involved in the biosynthesis of the triacylglycerol molecule are also used for the formation of the phosphoglycerides and the only unique reaction is the last step in the process (2), i.e., the acylation of the diacylglycerol molecule to form triacylglycerol. This step is catalyzed by the enzyme diacylglycerol:acyltransferase (DGAT, EC 2.3.1.20). Results by Mayorek, Grinstein, and Bar-Tana (3) indicate that this unique last step is rate-limiting in the biosynthesis of the triacylglycerol. On the other hand Brindley (4) and his co-workers have presented evidence that the dephosphorylation of phosphatidic acid could serve as a step in which hormones and fatty acids may influence the rate of triacylglycerol (and phosphoglyceride) formation.

The triacylglycerol biosynthesis that occurs in the liver is of great importance for the secretion of the apoB-100-containing lipoproteins. Thus it has been shown that an increased biosynthesis of triacylglycerol in the cell is a major stimuli for apoB-100 secretion (5, 6).

The assembly of these lipoproteins appears to take place in regions of the ER membrane that are rich in DGAT activity (7) and the importance of the enzyme for the assembly and secretion of lipoproteins is further underlined by the observation (8) that the inhibitory effect of n-3 fatty acids on the secretion of triacylglycerol-rich lipoproteins has been suggested to be caused by an inhibition of the DGAT activity.

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellar concentration; DGAT, diacylglycerol:acyltransferase (EC 2.3.1.20); DOC, sodium deoxycholate; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.05% Tween 20; PMSF, phenylmethylsulfonyl fluoride; SD, standard deviation; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline with 0.1% Tween 20.

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Some attempts have been made to isolate the enzyme DGAT. A 9-fold increase in the specific activity has been achieved by gradient ultracentrifugation after detergent treatment of rat liver microsomes (9), and a 145-fold purification was obtained from the intestine after solubilization in taurocholate followed by phenyl Sepharose chromatography (10). In both cases only partial purifications were obtained.

Purification of the enzyme from soybean cotyledons has also been reported (11). Analyses of these preparations by electrophoresis in polyacrylamide gels containing SDS suggested that the enzyme had a subunit structure.

In this report we present a method to isolate DGAT from rat liver microsomes to near homogeneity.

MATERIALS

Superose 6 prep grade, FPLC columns, Sephadex G 25, Q Sepharose fast flow, Sephacryl S-200 HR, CNBr-activated Sepharose 4B, Protein G Sepharose fast flow and pre-packed disposable PD-10 columns were purchased from Pharmacia (Uppsala, Sweden); Chemibond® was from Chemicon International Inc. (Temecula, CA); palmitoyl CoA, L-phosphatidylcholine (from egg yolk), 1,2-dioleoyl-*sn*-glycerol, L-phosphatidyl-L-serine, and pepstatin A were from Sigma (St. Louis, MO). Sucrose was purchased from International Biotechnology Inc. (New Haven, CT); [1-^{14}C]palmitoyl CoA, rainbow markers (14,300–200,000) and blotting detection kit for mouse antibodies were from Amersham Int. (Amersham, United Kingdom). Ready Safe® was from Beckman (Fullerton, California); pre-coated TLC plates with Silica gel 60 were from Merck (Darmstadt, Germany); mini-PROTEAN II Ready Gels and Silver Stain Plus Kit were from Bio-Rad (Richmond, CA); BCA protein Assay Reagent was from Pierce (Rockford, IL). Trioleate, calpain inhibitor I and calpain inhibitor II were purchased from Boehringer Mannheim (Mannheim, Germany); microtiter plates PVC M 24 were from Dynatech Labor Inc. (Chantilly, VA); urease-conjugated sheep anti-mouse Ig fraction was from Sera-lab Limited (Crawley Down, England).

Millex AA filter 0.8 μm, Millex GV filter 0.22 μm, and the Milli-Q UF plus equipment (used for the purification of all water that was used in the experiments) were from Millipore Intertech (Bedford, MA).

METHODS

Isolation of rat liver microsomes

The method described earlier (5) for cells was scaled up and modified to make it possible to handle livers from 50 Sprague-Dawley rats.

The rats were killed by decapitation and the livers were removed and washed with 3 mM imidazole, pH 7.4, with 125 mM sucrose. The livers were cut into small pieces and homogenized in 3 mM imidazole, pH 7.4, with 125 mM sucrose (20 ml buffer/liver) by five strokes with a rotating Teflon pestle in a glass homogenizer. The homogenate was centrifuged for 10 min in a Beckman J-21 centrifuge at 1700 rpm and +4°C using a JA-14 rotor. The pellet was washed with the imidazole buffer (4 ml buffer/liver) under the same conditions and the combined supernatants were centrifuged for 20 min in a Beckman J-21 at 13,000 rpm and +4°C using a JA-14 rotor, and the obtained supernatant was ultracentrifuged for 63 min at +4°C and 35,000 rpm in a Beckman Ti 50 rotor. The pellet was recovered and suspended (at a concentration of approximately 30 mg microsomal protein/ml) in 3 mM imidazole, pH 7.4, with 125 mM sucrose and homogenized with 17 strokes in a Dounce homogenizer. The obtained microsomes were frozen in aliquots of 120 mg and kept at −80°C until used.

Detergent treatment of the microsomes

Microsomes (120 mg) (measured as protein) were diluted to 30 ml with 50 mM Tris-HCl, pH 7.5, with 300 mM sucrose and 1.25 mM EDTA. The following protease inhibitors were used: calpain inhibitor I (17 μg/ml), calpain inhibitor II (7 μg/ml), and 1 μM pepstatin A. Sodium deoxycholate (DOC) was added to a final concentration of 1 mM (i.e., 0.1 mg/mg microsomal protein) (12). The mixture was left on ice for 60 min and was then centrifuged in a Beckman Ti 90 rotor at 40,000 rpm and +4°C for 60 min. The supernatant was removed and the tube and the pellet were carefully washed twice with 2 ml ice-cold 50 mM Tris-HCl, pH 7.5, with 300 mM sucrose and 1.25 mM EDTA. To each pellet (recovered from 50 mg microsomes) was added 2 ml of 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg DOC/ml, calpain inhibitor I and II (17 and 7 μg/ml), and 1 μM pepstatin A.

The pellet was first dispersed by forcing it through a 1-ml tip of an Eppendorf pipette approximately 10 times and then subjected to short (1–2 sec long) sonications in a MSE soniprep 150 at the following setting; 6, 10, 14, 18, 22, 26, and 30 microns. Four sonications were carried out at each setting.

CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; 5 mg/ml) was added to the solution and it was again subjected to sonication as described above at the following settings; 14, 22, 26, and 30 microns. The obtained suspension/solution was filtered through a 0.22-μm filter.

Diacylglycerol:acyltransferase assay (DGAT)

1,2-Dioleoyl-*sn*-glycerol was dispersed into portions enough for five assays and stored under nitrogen in sealed

glass ampules at −80°C. The storage time was usually below 30 days.

1,2-Dioleoyl-*sn*-glycerol (1 mg/assay) was dissolved in chloroform and mixed with 0.8 mg phosphatidylcholine and 0.8 mg phosphatidylserine and dried under nitrogen; 0.4 ml of 10 mM Tris-HCl, pH 8.0, was added and the mixture was sonicated for 60 sec on ice (using an MSE 8 ultrasonic at maximal setting).

Palmitoyl CoA (final concentration in assay 0.2 mM) and 0.188 μCi [1-^{14}C]palmitoyl CoA were added to the substrate, and the mixture was vortexed until all palmitoyl CoA had been solubilized.

The assay system contained 0.4 ml of the substrate solution, 0.150 ml of a 1.4 M solution of magnesium chloride, 0.6 ml 10 mM Tris-HCl, pH 8.0, and fatty acid-free bovine serum albumin (BSA) to a final concentration of 1 mg/ml. Fifty μl of the enzyme source was added to the assay simultaneously with the substrate solution, and the mixture was vortexed.

After incubation at 37°C, the assay system was transferred to 6 ml of chloroform–methanol 1:1 containing 0.1 mg trioleate, and the formed triacylglycerols were extracted in a two-phase system (13). In short, 4 ml of chloroform was added and the tubes were shaken and stored at −20°C for 10–12 h. Two ml of an acidified solution of sodium chloride (17 mM NaCl and 1 mM of H_2SO_4) was added, the tubes were again shaken and then centrifuged at 2500 rpm for 10 min at room temperature in a Beckman CPR centrifuge.

The upper phase was removed and the lower phase was recovered and evaporated to dryness under nitrogen.

The sample was dissolved in chloroform and subjected to thin-layer chromatography in chloroform–acetic acid 96:4. This chromatography gave a good separation be-

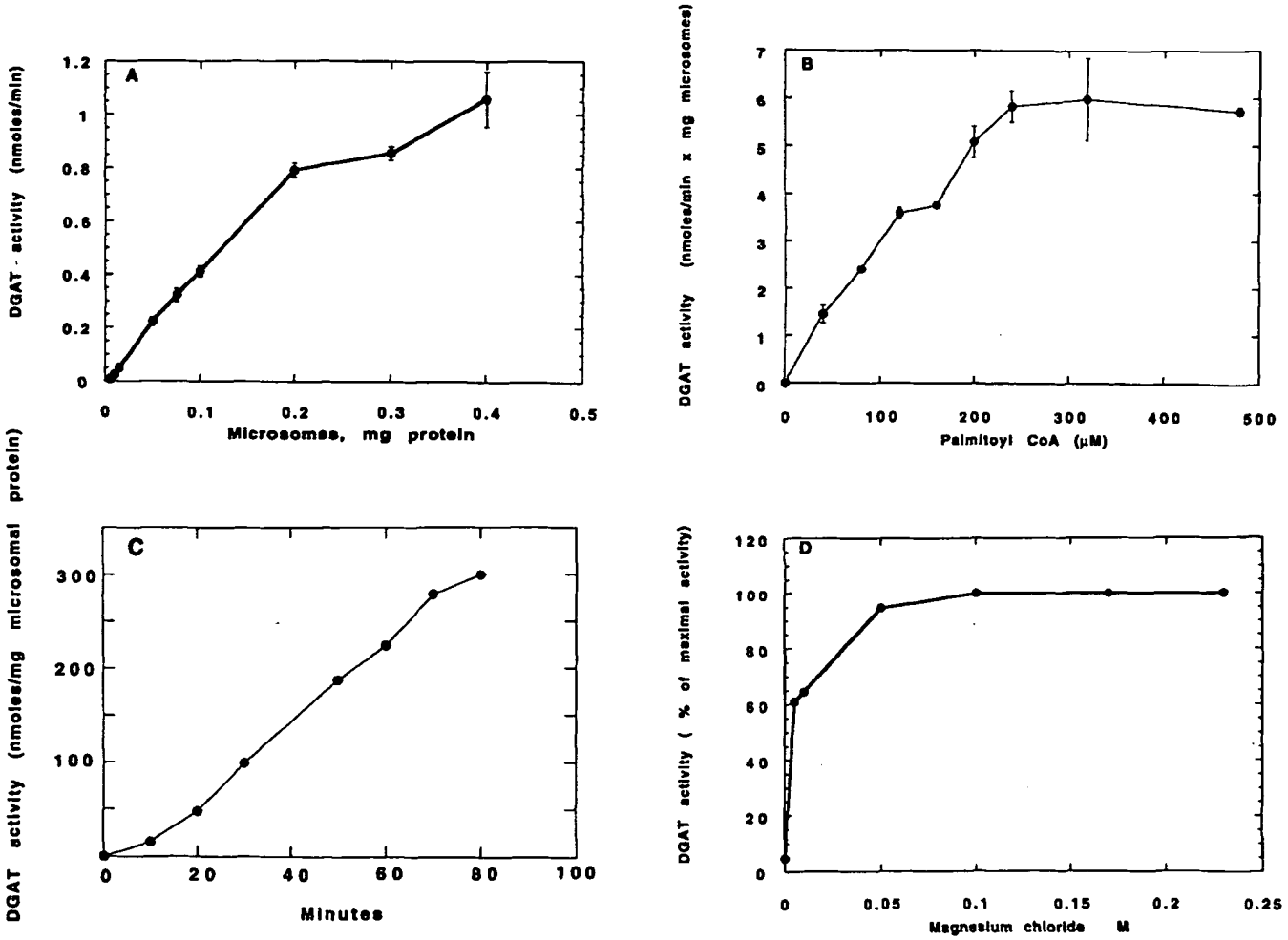

Fig. 1. A: The relation between the amount of microsomal protein and the DGAT activity (nmole formed triacylglycerol/min). Results are given as mean ± SD, n = 5. B: The relation between the DGAT activity (nmole formed triacylglycerol/min and mg protein) and the concentration of palmitoyl CoA in the assay. Results are given as mean ± SD, n = 5. C: The relation between the DGAT activity (nmole formed triacylglycerol/mg microsomal protein) and the length of the incubation. Results are given as mean of three experiments. D: The relation between the DGAT activity (given as % of maximal activity) and the concentration of magnesium chloride in the assay. Results are given as mean of three experiments.

tween triacylglycerol (R_f value: 0.78) and diacylglycerol (R_f value: 0.43), free fatty acids (R_f value: 0.47) and acyl-CoA (which remained on the application point). The spot corresponding to triacylglycerol was identified after staining in iodine vapor, scraped off, extracted in 1 ml chloroform, and counted in Ready Safe in a Beckman LS 6000 TA Liquid Scintillator. Correction for quenching was made.

The assay was totally dependent on microsomes or solubilized DGAT and there was a linear relation between the amount of triacylglycerol formed and the mass of microsomal protein between 0.05 mg and 0.2 mg (**Fig. 1A**).

The assay was dependent on both acyl-CoA (Fig. 1B) and diacylglycerol. When microsomes were used as the enzyme source, we could estimate that less than 2% of the activity was not dependent on exogenous diacylglycerol. The activity determined in isolated microsomes varied between 2 and 6 nmol/min and mg microsomal protein which was within the range reported by other authors (9).

Time course showed a linearity between 20 and 70 min (Fig. 1C) whereas the curve showed a tendency to level off after 70 min incubation. We routinely used a 60-min incubation.

The reaction was totally dependent on $MgCl_2$ and a reaction maximum was reached at a concentration of 50 mM (Fig. 1D). We have chosen to use a higher concentration, i.e., 150 mM.

The within assay variation was 6% (n = 28).

To determine the hydrolysis of acyl-CoA, the samples were incubated with 0.2 mM palmitoyl CoA together with 0.04 μCi [1-^{14}C]palmitoyl CoA, in 10 mM Tris-HCl, pH 8.0, with 150 mM $MgCl_2$ and 1 mg/ml BSA for 60 min. Palmitic acid was added as carrier and the lipids were extracted and analyzed by TLC as described above. The spot corresponding to palmitic acid was scraped off and the radioactivity was counted as described above. Correction for quenching was made.

Chromatography on Superose 6

The Superose 6 column (Pharmacia HR 16/50) was equilibrated with 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml DOC, and 1 μM pepstatin A. One ml of the filtered detergent-treated microsomes was applied to the column and the chromatography was carried out at a flow rate of 1 ml/min using an LKB 2150 HPLC pump (Pharmacia Uppsala, Sweden). The absorbance at 280 nm was measured in an LKB 2158 UVICORD SD (Pharmacia Uppsala, Sweden) and fractions of 1 ml were collected. The chromatography was carried out at room temperature but the fractions containing the DGAT activity were rapidly combined and placed on ice.

Q Sepharose and immunoaffinity chromatography

The DGAT-containing fractions from the Superose 6 chromatography were combined and dithiothreitol (DTT) was added to a final concentration of 2 mM. This was essential for the stabilization of the activity during the following steps in the isolation procedure. The combined fractions were chromatographed on a 2-ml column of Q Sepharose equilibrated with 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml DOC, 5 mg/ml CHAPS, 2 mM DTT, and 1 μM pepstatin A (this buffer is referred to as buffer A in the following). The unretained material (eluted with the same buffer) was collected on ice.

The DGAT-containing fraction from the Q Sepharose was chromatographed on an immunoadsorber based on a monoclonal antibody (see below). The antibody was purified from the hybridoma culture medium by chromatography on Chemibond® and immobilized on Sepharose (see below). The adsorber (volume 2–3 ml) was poured on top of a Sephadex G 25 column (volume 3 ml) and equilibrated with buffer A. The unretained material was eluted with 10 column volumes of this buffer. To desorp the retained proteins we applied 0.5 ml of 3 M sodium thiocyanate in buffer A to the column. The column was then eluted with buffer A. Fractions of 0.15–0.3 ml were collected on ice and analyzed for the presence of DGAT activity. The analysis was carried out within an hour.

Chromatography on Protein G Sepharose and Chemibond®

The DGAT-containing fractions were chromatographed on a 0.6-ml column of Protein G Sepharose or Chemibond® equilibrated with buffer A. The unretained material was collected in 0.3-ml fractions on ice.

Immunoglobulins from the hybridoma supernatant were isolated by chromatography on Chemibond® using the protocol recommended by the manufacturer, i.e., 50 ml of hybridoma supernatant was applied to a 1-ml column of Chemibond® equilibrated with phosphate-buffered saline (PBS) pH 7.4. The column was washed with PBS until all the phenol red dye was removed from the column (50–100 ml). The bound antibodies were eluted with 0.05 M sodium acetate, pH 2.8, precipitated with ammonium sulfate and resolubilized in 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl.

Isolation of monoclonal antibodies

The base for the strategy to isolate a monoclonal antibody to DGAT was the observation from radiation inactivation studies done by other investigators (14) that DGAT had a molecular mass of about 70 kDa. The aim was, therefore, to isolate a fraction enriched in microsomal proteins about 70 kDa and to use that fraction for immunization and primary screening of the obtained hybridomas. Several methods have been tried but the following turned out to be useful.

The rat liver microsomes were diluted with 3 mM imidazole, pH 7.4, containing 125 mM sucrose to a final

concentration of 0.6 mg/ml (measured as protein). In general, we started each isolation with 1200 mg microsomes. Sodium carbonate (1 M) was added to a final concentration of 0.1 M and a pH of 10.3, and the solution was incubated on ice for 30 min with intermittent stirring. This was followed by centrifugation for 60 min in a Beckman J-21 centrifuge at 10,000 rpm in a JA-14 rotor at +4°C.

The supernatant (2400 ml) was directly loaded onto a 170 ml Q Sepharose column (5 × 8 cm) equilibrated with 50 mM Tris, pH 10.3, with 300 mM sucrose, 5 mM $MgCl_2$, 1.25 mM EDTA, 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin (this buffer will be referred to as buffer B in the following) and chromatographed at a flow rate of 60 ml/h. The unretained fraction was collected and the column was washed with two volumes of buffer B. The chromatography was carried out at +4°C.

The unretained fraction was lyophilized and resolubilized in approximately 500 ml water containing 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. The sucrose concentration of the resolubilized sample was kept between 40 and 60% w/v.

The solubilized material was homogenized by 15 strokes in a Dounce homogenizer with a tight-fitting glass pestle, and filtered through a series of glass filters with decreasing pore size (Jena Glas G2, G3, and G4), followed by two passages through a 3-μm Millipore filter and finally one passage through a 0.8-μm filter.

The filtered solution was chromatographed on a 5 × 110 cm column of Sephacryl S-200 HR, equilibrated with buffer B. The chromatography was carried out at +4°C at a flow rate of 40–60 ml per h and the absorbance at 280 nm was constantly monitored by a UVICORD S (Pharmacia LKB Uppsala, Sweden). Based on the obtained chromatogram (Fig. 2A), fractions of 50–100 ml were collected, dialyzed against water, and lyophilized. The fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS. In this way we obtained a fraction that was enriched in proteins with a molecular mass between 50 and 80 kDa (Fig. 2B). This fraction was used for immunization of Balb C mice.

A primary injection was followed by a booster injection after 5, 7, and 9 weeks. Spleen cells were hybridized with Sp/2/O-Ag-14 cells and cultured in the presence of mouse peritoneal macrophages as feeder cells.

The clones were tested with ELISA (see below) against the fractions used for immunization. Positive clones were recloned by the method of single-cell cloning (6). The primary screening of the clones, obtained after recloning, was carried out with ELISA against the fraction used for immunization as well as against the DGAT-containing fraction from the Superose 6 column (see Results). Positive clones were analyzed with Western blots to identify clones that reacted with a protein with a molecular mass of 60–80 kDa, i.e., similar to that determined for DGAT by radiation inactivation studies (14). Such clones were

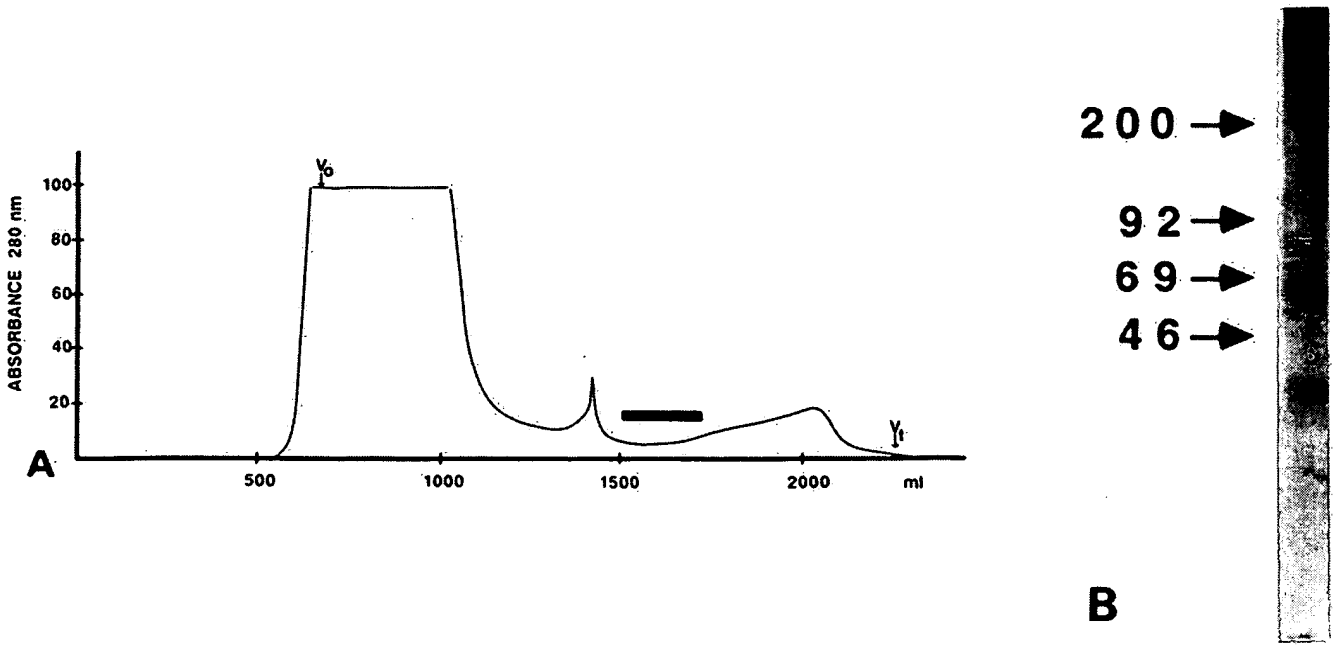

Fig. 2. A: Chromatography of the unretained fraction from the ion exchange (Q Sepharose) chromatography on Sephacryl S-200 HR equilibrated with 50 mM Tris, pH 10.3, with 300 mM sucrose, 5 mM $MgCl_2$, 1.25 mM EDTA, 1 mM PMSF, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. The chromatography was carried out at +4°C with a flow rate of 60 ml/h. The absorbance at 280 nm was measured continuously; V_o, void volume; V_t, total volume. The fraction that was used for immunization is indicated with a bar in the chromatogram. B: Electrophoresis in 4–20% polyacrylamide gradient gels containing SDS of the fraction from the Sephacryl S-200 HR chromatography that was used for immunization of Balb C mice. (The fraction is indicated with a bar in Fig. 2A.) Arrows indicate the migration of the 200, 92, 69, and 46 kDa standards. The gel is silver stained.

tested for their ability to inactivate DGAT and, after immobilization, for their ability to retain the DGAT activity.

In order to prepare immunoadsorbers, the immunoglobulin (Ig) fraction was recovered from the culture medium by chromatography on Chemibond® followed by precipitation with ammonium sulfate (see above). The Ig fraction was solubilized in 0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl and coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer (Pharmacia Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was carried out on microtiter plates (PVC M 24). The wells were coated with either the fraction from the Sephacryl S-200 HR chromatography or the DGAT-containing fraction from the Superose 6 chromatography.

The fraction from the Sephacryl S-200 HR column was dialyzed against a 50 mM sodium carbonate buffer, pH 9.6, with 3 mM sodium azide before it was used to coat the wells, while the DGAT-containing fraction from the Superose 6 chromatography was desalted on a PD 10 column, equilibrated with the same buffer, before the fraction was used for the coating. The coating was carried out overnight at +4°C, and was followed by three washes with PBS containing 0.05% Tween 20 (PBS-T). Residual binding sites in the wells were blocked by incubation with PBS containing 3% BSA for 30 min at room temperature.

Incubation with the hybridoma supernatant was carried out for 1 h at 37°C followed by three washes with PBS-T. To detect the bound antibodies, we used a urease-conjugated sheep anti-mouse Ig fraction in PBS-T containing 0.25% BSA. The incubation was carried out for 1 h at 37°C and was followed by six washes with PBS-T and three washes with water. After development, the microtiter plates were read at 570 nm in a microplate reader model 450 (Bio-Rad).

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out either in 4–20% gradient gels (using Mini-PROTEAN II Ready Gels, Bio-Rad), that had been pre-run for 30 min or in 3–15% gradient gels. To avoid degradation during dialysis, lyophilization, or concentration, we analyzed the material as it was eluted from the immunoadsorber. SDS (final concentration of 2.3%) and DTT (final concentration 75 mM) were added to the sample and it was boiled for 5 min. The electrophoresis was carried out at 20 mA/gel with the equipment placed in an ice-bath. The gels were stained with silver, using the Silver Stain Plus Kit, as recommended by the manufacturer (Bio-Rad). Rainbow molecular weight markers were used to standardize the gels. This kit contains prestained myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

Immunoblots were carried out on a Multiphore II (Pharmacia LKB Uppsala, Sweden) as recommended by the manufacturer. The blots were blocked with 5% non-fat dry milk in 20 mM Tris-HCl, pH 7.6, containing 137 mM NaCl and 0.1% Tween 20 (TBS-T) overnight. This was followed by a 1-h incubation with the antibody in 20 mM Tris-HCl, pH 7.6, containing 137 mM NaCl (TBS) with 5% non-fat dry milk. The blots were washed with TBS-T for 2 × 1 and 3 × 5 min. To detect the bound antibody we used a biotinylated antibody to mice Ig followed by streptavidin-conjugated alkaline phosphatase, using the Amersham blotting detection kit for mice (i.e., monoclonal) antibodies. After the incubation with the second antibody, the filter was washed with TBS-T for 2 × 1, 2 × 15, and 1 × 5 min. The incubation with the alkaline phosphatase was followed by washes with TBS-T for 2 × 1 and 3 × 5 min. To control for unspecific binding, we used an irrelevant monoclonal antibody, i.e., a monoclonal antibody obtained from Balb C mice that had not been immunized with rat proteins.

Protein determination

The protein content of microsomes, solubilized microsomes, and fractions from the Superose 6 column was determined by the BCA method, using BSA as standard. Both DOC and CHAPS influence this method, but this influence could be overcome by diluting the sample 200-fold with 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, and 1% Triton X-100. To estimate the protein content in the immunoaffinity-purified enzyme, we scanned the silver-stained SDS-polyacrylamide gels. The electrophoresis and silver staining were carried out as described above. As standards we used a dilution series of BSA run in duplicate at the same time as the samples. The gels were scanned at 500 nm with a dual-wavelength flying-spot Shimadzu CS-9000 scanner equipped with a Shimadzu DR-13 computer unit. A linear relation between the absorbance and the amount of protein was obtained between 0.025 and 0.1 μg BSA. All samples were within this linear part of the curve.

RESULTS

Solubilization and gel chromatography of diacylglycerol:acyltransferase (DGAT)

The solubilization procedure started with an extraction of luminal and loosely bound proteins from the microsomes by sodium deoxycholate (DOC) below the critical micellar concentration (CMC). During this extraction 3% of the DGAT activity and all of the acyl-CoA hydrolase activity were recovered in the supernatant after

TABLE 1. Recovery of protein and DGAT activity during purification of the enzyme

	Recovery DGAT Total	Activity In Step	Recovery Protein	DGAT Activity	Purification
	%	%	%	*nmol/min × mg protein*	*fold*
Microsomes	100	100	100	2	1
Solubilized microsomes	46	46	21	5	2.5
Solubilized microsomes after filtration	37	83	20	5	2.5
Superose 6 chromatography	17	45	5	7	3.5
Q Sepharose and immunoaffinity chromatography	4	22[a]	0.0045	830	415

Results are mean of three different experiments.
[a]The recovery of the applied activity in the unretained fraction from the Q Sepharose was 45% and in the retained fraction from the immunoaffinity column was 49%.

ultracentrifugation. Thus no hydrolase activity could be detected in the membrane pellet or in any of the fractions recovered from gel chromatography (described below).

The membrane pellet was sonicated in DOC and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at concentrations above the CMC, and filtered through a 0.22-μm filter. Thirty seven percent (**Table 1**) of the initial activity present in the total microsome was recovered in the filtrate. The filtrate was chromatographed on a Superose 6 column, from which the DGAT activity was eluted in a high molecular weight peak (**Fig. 3**). Forty five percent (Table 1) of the applied activity was recovered during this chromatography; this corresponded to a recovery of 17% of the total activity present in the microsomes.

The DGAT-containing fraction from the Superose 6 column chromatography was heterogeneous as judged from its appearance on polyacrylamide gel electrophoresis (Fig. 4, lane 2).

Isolation of a monoclonal antibody that could be used for immunoadsorbtion of DGAT

As a result of three different hybridizations, we found one clone that fulfilled the criteria that were set up. Thus this clone reacted on ELISA with the fraction used for immunization as well as with the DGAT-containing fraction from the Superose 6 chromatography (**Fig. 5**). Moreover, it reacted with a 60 kDa protein on Western blot of solubilized microsomal proteins (**Fig. 6A**) as well as with the DGAT-containing fraction recovered from the Superose 6 column chromatography (Fig. 6B).

The monoclonal antibody did not react with rat serum or rat albumin (not shown).

The obtained clone produced an antibody of the IgG3 class. As the antibody did not inhibit the DGAT reaction (not shown) we used immunoaffinity chromatography in order to investigate its relation to the DGAT activity. The DGAT-containing fraction from the Superose 6 column was chromatographed on a 2-ml immunoaffinity column

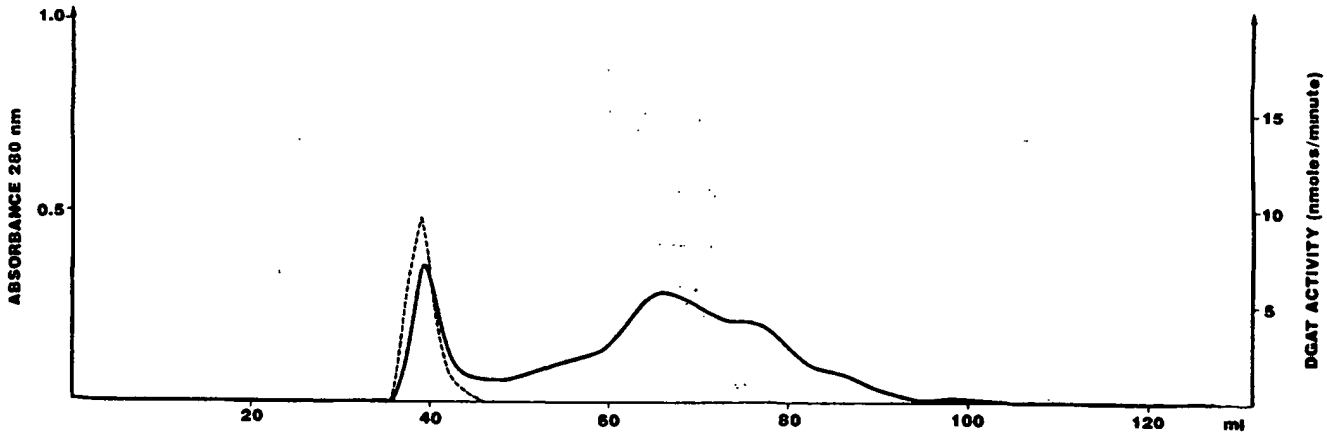

Fig. 3. Gel chromatography of detergent-treated microsomes on Superose 6. One ml of the filtered detergent-treated microsomes was applied to a 1.6 × 45 cm column in 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1.25 mM EDTA, 1.6 mg/ml sodium deoxycholate, and 1 μM pepstatin A. The column was eluted at a rate of 1 ml/min. The effluent was constantly monitored for the absorbancy at 280 nm (smooth line) and fractions of 1 ml were collected and analyzed for DGAT activity (nmole formed triacylglycerol/min, dashed line).

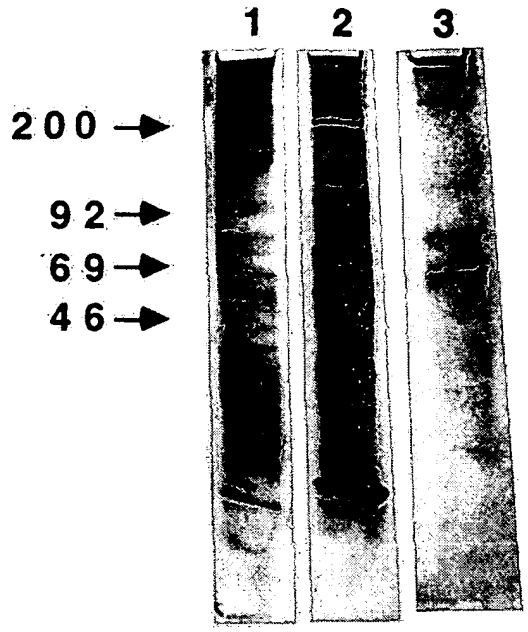

Fig. 4. Electrophoresis in 4–20% polyacrylamide gradient gels containing SDS of total rat liver microsomes (lane 1); the DGAT-containing fraction from the Superose 6 column (lane 2); and the retained (DGAT-containing) fraction from the immunoaffinity chromatography (lane 3). The arrows mark the migration of the 200, 92, 69, and 46 kDa standards.

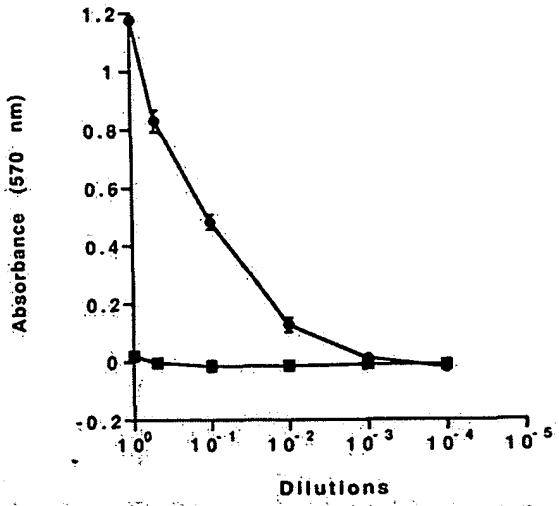

Fig. 5. The reaction between the DGAT-containing fraction from the Superose 6 chromatography and different dilutions of the hybridoma supernatant (●) as well as an irrelevant antibody (■). The reaction was investigated with an ELISA system in which the wells were coated with the DGAT fraction from the Superose 6 chromatography. The bound antibody was detected with a goat anti-mouse antibody coupled to urease. Results are given as mean ± SD, n = 5.

(a total activity of 1 nmol formed triacylglycerol/min was applied to each column). 93 ± 6% (mean ± SD, n = 4) of the applied activity was retained by the immunoadsorber.

The desorption of the enzyme was carried out with sodium thiocyanate which, however, inhibited the activity (not shown). In order to limit this inhibition, we poured the immunoadsorber on top of a small column of Sephadex G-25; thus the desorbed enzyme was immediately separated from the bulk of the sodium thiocyanate. Using this method we could recover 44 ± 6% (mean ± SD, n = 5) of the applied DGAT activity from the immunoaffinity column after the desorption.

The results indicated that the monoclonal antibody could be used to isolate the enzyme by immunoaffinity chromatography.

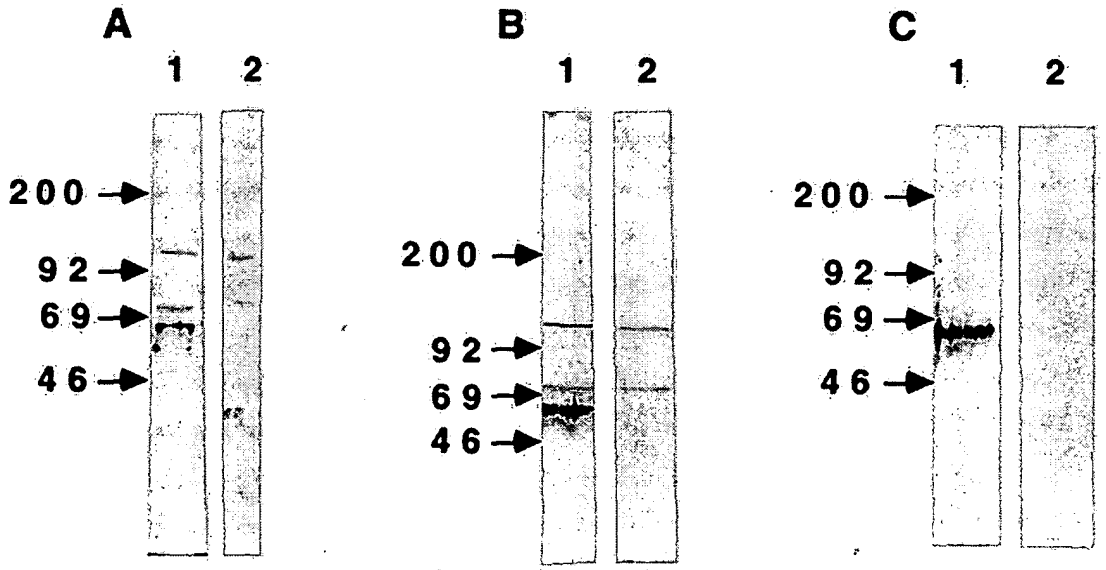

Fig. 6. Immunoblot of rat liver microsomes (0.23 mg/slot) (A), the DGAT-containing fraction from the Superose 6 chromatography (0.05 mg/slot) (B), and immunopurified DGAT (C). The blots were probed with the monoclonal antibody to DGAT (lane 1) or an irrelevant monoclonal antibody (lane 2). The electrophoresis was carried out in 3–15% polyacrylamide gradient gels containing SDS. The bound antibodies were detected by a biotinylated sheep anti-mouse antibody followed by incubation with streptavidin-conjugated alkaline phosphatase. The arrows mark the migration of the 200, 97, 69, and 46 kDa standards.

Q Sepharose and immunoaffinity chromatography of the DGAT-containing fraction from the gel chromatography

The DGAT-containing fraction from the gel chromatography was first chromatographed on a Q Sepharose from which 74 ± 14% (mean ± SD, n = 5) of the recovered activity was eluted in an unretained fraction. Forty five percent of the applied activity was recovered in this unretained fraction.

The unretained fraction from the Q Sepharose chromatography was subjected to immunoaffinity chromatography on the obtained monoclonal antibody and the retained fraction containing the DGAT activity was analyzed by electrophoresis in polyacrylamide gradient gels (PAG) containing SDS. The gels showed, after silver staining, a marked enrichment in a protein with a molecular mass of 60 kDa as well as a 77 kDa protein (Fig. 4, lane 3). These proteins were only present in the fractions that contained the DGAT activity. In addition to these proteins we could detect trace amounts of proteins with molecular masses of 200 and 120 kDa. A pre-run of the fraction on an adsorber, containing an irrelevant antibody before the immunoaffinity chromatography, did not change the electrophoresis pattern (not shown). We also chromatographed the retained fraction from the immunoaffinity chromatography on a Protein G Sepharose column and on a Chemibond® column to remove immunoglobulins that might have leaked from the adsorber. This did not influence the electrophoresis pattern (not shown).

The 60 and 77 kDa bands were also present after rechromatography on the immunoaffinity column (not shown).

Immunoblot showed that the monoclonal antibody reacted with the 60 kDa proteins but not with the 77 kDa protein (Fig. 6C). As discussed above, the antibody also reacted with a 60 kDa protein on Western blot of both solubilized microsomes and of the DGAT-containing fraction recovered from the Superose 6 column (Fig. 6, A and B). In addition to this reaction, there were proteins present in the solubilized microsomes as well as in the DGAT-containing fraction from the Superose 6 chromatography that reacted unspecifically with immunoglobulins or were lit up by the second antibody (i.e., the sheep anti-mice Ig). These proteins were not present after the immunoaffinity purification (Fig. 6C) and their nature has not been investigated further.

DISCUSSION

We have developed a method to purify diacylglycerol: acyltransferase (DGAT) from rat liver microsomes to near homogeneity. The method was based on the solubilization of the DGAT activity from the microsomes by sonication in sodium deoxycholate (DOC) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) followed by gel chromatography and immunoaffinity chromatography, utilizing a monoclonal antibody.

Radiation inactivation studies (14) indicate that DGAT has a molecular mass of 72 ± 4 kDa; we therefore developed a method to isolate a fraction enriched in microsomal proteins with molecular masses between 50 and 80 kDa and raised monoclonal antibodies to this fraction. One antibody was obtained that reacted with a 60 kDa protein within the microsomal proteins as well as in DGAT-containing fractions from gel chromatography. The antibody did not inhibit DGAT but could, when used in immunoaffinity experiments, quantitatively remove the DGAT activity from a solution. Moreover, 44% of the applied activity could be recovered after desorption of the enzyme from the immunoaffinity column. The discrepancy between the adsorbed and recovered DGAT activity is not unexpected and could most likely be explained by: *i)* a denaturation of the enzyme during the adsorption–desorption procedure; *ii)* an inhibition of the enzyme activity by the sodium thiocyanate used for desorption (although attempts were made to rapidly separate the enzyme from the sodium thiocyanate, it is most likely that this separation is incomplete and that the thiocyanate contaminates the last portion of the enzyme peak) or *iii)* perhaps a failure to desorb all enzyme.

The purification method gave rise to a 415-fold increase in the specific activity. It should be pointed out that it is possible that this is an underestimation because, as discussed above, the enzyme may to some degree be inactivated during the immunoaffinity chromatography.

The antibody recognized a protein with an estimated molecular mass of 60 kDa on Western blot of microsomal proteins. A protein of the same size was also highly enriched in the retained fraction from the immunoaffinity chromatography that contained the enzyme activity. In addition, this fraction contained a 77 kDa protein. The relation between these two proteins is not clear. The observation that the epitope for the antibody resides on the 60 kDa protein and that the two proteins are desorbed together from the immunoaffinity column, even after washes of the column with detergent-containing buffers prior to the desorption, indicates a strong interaction between the two components.

The possibility that the two proteins are generated by proteolysis should be considered. The observation that the monoclonal epitope is confined to the 60 kDa protein argues against the possibility that this protein is generated from the 77 kDa protein by proteolysis. It is, however, possible that the 60 and 77 kDa proteins are generated by proteolysis of a larger protein. Thus, for example, a hinge region between two domains may provide a site for such a proteolysis. However, one would have anticipated that at least a portion of the tentative full-length protein could be detected by the antibody during the Western blot analysis.

It is possible that the 77 kDa protein represents a unrelated contaminant. If this is the case our results suggest that the 77 kDa protein is either derived from the immunoadsorber or is a protein that has a high unspecific affinity for immunoglobulins. Against these possibilities argue the results from the immunoblotting experiment (Fig. 6C) which indicated that the protein neither reacted with the sheep anti-mice Ig (used as the second antibody) nor with unspecific immunoglobulins; moreover, neither chromatography on an adsorber with an irrelevant antibody nor chromatography on protein G and/or Chemibond® removed the 77 kDa protein.

A final possibility that has to be considered is that DGAT has a subunit structure. In fact, the failure of the monoclonal antibody to inhibit the enzyme activity prevents us from concluding that the active site is present on the 60 kDa protein. However, a protein with a molecular mass close to 140 kDa would not be supported by the results from the radiation inactivation studies (14), while both molecular masses of 60 kDa and 77 kDa would be compatible with these observations.

A subunit structure has been suggested for the enzyme that was isolated from soybean cotyledons (10), however none of the identified subunits of this enzyme appear to correspond to the proteins reported in this paper. Moreover, the enzyme isolated from the liver microsomes appeared to have much (near 1000-fold) higher specific activity than the enzyme isolated from the cotyledons.

The DGAT activity is eluted in a high molecular weight fraction from the Superose 6 column. Even if the enzyme consists of two subunits with a combined molecular mass of near 140 kDa, this could not explain its appearance on gel chromatography, suggesting that the enzyme occurs in a high molecular weight complex when extracted from the microsomes. Indeed, results from studies in rat intestine indicate that the whole triacylglycerol synthetase complex could be solubilized in the presence of taurocholate (10).

It has recently been demonstrated that the microsomes of rat intestine contain an enzyme that forms triacylglycerol by transacylation (15). This transacylase was not dependent on acyl-CoA, and it differed in molecular weight from the enzyme isolated in this study.

Rat liver contains an acyl-CoA hydrolase activity that generates fatty acids from acyl-CoA. The thin-layer chromatography system used in this study does separate fatty acids from triacylglycerol. Moreover, the acyl-CoA hydrolase appeared to be completely removed during the first extraction of the microsomes as we could not detect the activity in the membrane pellet or in the fractions from the gel column.

In conclusion, the results presented in this paper suggest that DGAT consists of a 60 kDa protein and perhaps also a 77 kDa protein. Further studies including cloning and expression of the enzyme are needed to unequivocally clarify the relation between these proteins.

The excellent technical assistance of Margareta Evaldsson is greatly acknowledged. We would like to thank Dr. Eva Sjögren-Jansson for expert advice and help during the preparation of the monoclonal antibody. This work was supported by grants 7142 and 8862 from the Swedish Medical Research Council, the Swedish National Association against Heart and Lung Disease, The Oleo-Margarine Foundation of Nutritional Research, King Gustav V's Foundation, The Swedish National Board for Technical Development, The Insulin Foundation, the Göteborg Medical Society, Torsten and Ragnar Söderbergs Foundation, Ulf Widenberg Foundation, and British Biotechnology Limited.

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Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis

(fatty acid/cloning/expressed sequence tag/glycerolipid)

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ABSTRACT **Triacylglycerols are quantitatively the most important storage form of energy for eukaryotic cells. Acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the terminal and only committed step in triacylglycerol synthesis, by using diacylglycerol and fatty acyl CoA as substrates. DGAT plays a fundamental role in the metabolism of cellular diacylglycerol and is important in higher eukaryotes for physiologic processes involving triacylglycerol metabolism such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation. DGAT is an integral membrane protein that has never been purified to homogeneity, nor has its gene been cloned. We identified an expressed sequence tag clone that shared regions of similarity with acyl CoA:cholesterol acyltransferase, an enzyme that also uses fatty acyl CoA as a substrate. Expression of a mouse cDNA for this expressed sequence tag in insect cells resulted in high levels of DGAT activity in cell membranes. No other acyltransferase activity was detected when a variety of substrates, including cholesterol, were used as acyl acceptors. The gene was expressed in all tissues examined; during differentiation of NIH 3T3-L1 cells into adipocytes, its expression increased markedly in parallel with increases in DGAT activity. The identification of this cDNA encoding a DGAT will greatly facilitate studies of cellular glycerolipid metabolism and its regulation.**

Acyl CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) is a microsomal enzyme that plays a central role in the metabolism of cellular glycerolipids (for reviews, see refs. 1 and 2). DGAT catalyzes the only committed step in triacylglycerol synthesis by using diacylglycerol (DAG) and fatty acyl CoAs as its substrates. DAG used in the DGAT reaction can be derived from the hydrolysis of phosphatidic acid produced by the *de novo* synthesis pathway from glycerol-3-phosphate (Fig. 1). Alternatively, DAG can be derived from the esterification of monoacylglycerol (MAG), a pathway of importance in intestinal fat absorption (3), and from the hydrolysis of triacylglycerol or phospholipids. Inasmuch as DAG is a precursor for phospholipid synthesis and is an important signaling molecule that activates protein kinase C (4), DGAT activity potentially could regulate these cellular processes. Because of its role in triacylglycerol synthesis and energy storage, DGAT also may be involved in intestinal fat absorption (3), lipoprotein assembly and the regulation of plasma triacylglycerol concentrations (1, 5), fat storage in adipocytes (6), energy metabolism in muscle (7), milk production (1), and egg production, including mammalian oocytes (8). In plants, DGAT has an important function in the generation of seed oils (9). DGAT activity also has been found in *Mycobacteria* (10) and *Streptomyces* (11), and in the lipid bodies of fungi (12) and insects (13).

Although it has been partially purified (14, 15), DGAT has been difficult to isolate because it is an intrinsic membrane protein. Through homology searches of the expressed sequence tag (EST) databases by using coding sequences from acyl CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26), an acyltransferase that synthesizes cholesterol esters from cholesterol and fatty acyl CoA substrates (16), we identified an EST clone that shares homology with sequences in the ACAT C terminus. In this study, we demonstrate that this recently identified cDNA does not encode another ACAT, but in fact encodes a DGAT.‡‡ The expression of a mouse cDNA for this EST in insect cells resulted in high levels of a membrane-associated acyltransferase activity specific for DAG. This gene and its encoded activity then were characterized in detail.

MATERIALS AND METHODS

Cloning of DGAT cDNA. ESTs [accession nos. R07932 (human) and W10786 (mouse)] with sequence similarity to ACAT were identified from BLAST database searches. The 5′ end of the DGAT cDNA was obtained by using 5′ rapid amplification of cDNA ends (RACE) and a mouse spleen Marathon Ready cDNA library (CLONTECH). Sequences have been deposited in GenBank (accession no. AF078752).

Insect Cell Expression Studies. DGAT coding sequences with or without an N-terminal FLAG epitope (IBI/Kodak, New Haven, CT) (MGDYKDDDDG-, epitope underlined) were subcloned into pVL1392 (PharMingen). High titers of recombinant baculoviruses were obtained by cotransfection of baculovirus transfer vectors with viral BaculoGold DNA (PharMingen), followed by plaque purification and amplification in Sf9 cells [cultured in Grace's medium (Life Technologies, Grand Island, NY) and 10% fetal bovine serum]. H5 insect cells [cultured in serum-free Express-Five medium (Life

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Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; DAG, diacylglycerol; DGAT, acyl CoA:diacylglycerol acyltransferase; EST, expressed sequence tag; MAG, monoacylglycerol; MOI, multiplicity of infection.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF078752).

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‡‡Portions of this work were presented at the Experimental Biology meeting (San Francisco, 1998) and have been published in abstract form (34).

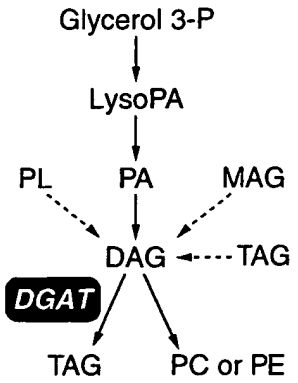

FIG. 1. Role of DGAT in glycerolipid metabolism. DAG used by DGAT potentially originates from hydrolysis of phosphatidic acid (PA), from the esterification of 2-monoacylglycerol (MAG), or from triacylglycerol (TAG) or phospholipid (PL) hydrolysis. The MAG pathway is thought to be especially important in enterocytes of the small intestine (3). P, phosphate; LysoPA, lysophosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Technologies)] were plated on day 0 (8.5×10^6 cells/100-mm dish) and infected on day 1 with high titers of virus at a multiplicity of infection (MOI) that was empirically determined. On day 3, cells were collected by centrifugation and washed twice with PBS. Cell pellets were homogenized by 10 passages through a 27-gauge needle in 0.1 M sucrose, 50 mM KCl, 40 mM KH_2PO_4, and 30 mM EDTA (pH 7.2). Total membrane fractions (100,000 × *g* pellet) were resuspended in the homogenization buffer and frozen (−80°C). Immunoblots of membrane proteins (75 μg) were performed with the anti-FLAG M2 mAb (IBI/Kodak).

For metabolic labeling, H5 insect cells were plated on day 0 (2.9×10^6 cells/60-mm dish) and infected on day 1 with high titers of viruses. On day 3, cells were washed and incubated in methionine- and cysteine-free medium (SF900 II, Life Technologies) for 2 h, followed by incubation in the same medium containing 715 μCi of [^{35}S]methionine and [^{35}S]cysteine (ProMix; Amersham; 1 Ci = 37 GBq) for 1 h. Cells were washed twice with PBS and collected by low-speed centrifugation. The cell pellet was resuspended in 0.5 ml of 50 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 (pH 7.4) and sonicated. Cellular proteins (100 μg) were analyzed by SDS/PAGE and autoradiography.

For ACAT assays, cell-membrane proteins (100 μg) were assayed by using [1-^{14}C]oleoyl CoA (51 mCi/mmol, Amersham) and cholesterol/egg phosphatidylcholine (PC) liposomes (molar ratio = 0.7) as described (17). In some assays, other acyl acceptors were substituted for cholesterol in the liposomes at a molar ratio of 0.2 (acceptor/egg PC). Incorporation of the [^{14}C]oleoyl group into products was assessed by TLC, followed by autoradiography. DGAT assays were based on assays optimized for rat liver (15, 18) (S.K.E., K. Pella, and S.R.L., unpublished data). The incorporation of [^{14}C]oleoyl CoA into triacylglycerol was measured under apparent V_{MAX} conditions by using exogenous DAG provided as DAG/egg PC liposomes (molar ratio ≈0.16). Cell-membrane proteins (20–25 μg) were assayed in 0.25 M sucrose, 1 mM EDTA, 150 mM $MgCl_2$, and 100 mM Tris·HCl (pH 7.5) containing 250 μg of BSA and 20 μg of DAG in liposomes and 5 nmol [^{14}C]oleoyl CoA (40,000 dpm/nmol) (final volume, 0.2 ml). Reactions were carried out for 5 min, and the products were analyzed as described (19). Similar assays were performed with 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol (53 mCi/mmol, Amersham) diluted to a final activity of 38,000 dpm/nmol with unlabeled 1,2-diacyl-*sn*-glycerol and unlabeled oleoyl CoA.

Relative triacylglycerol and DAG masses were determined by total lipid extraction of membranes or cells followed by TLC, iodine vapor visualization, photography of the plates, and densitometric analysis. Triolein standards were used to estimate the mass of triacylglycerols, and DAG units were estimated relative to one another. Triacylglycerol values were normalized to 1 for wild-type virus-infected cell membranes to correct for inter-experiment variability.

mRNA Expression. Human Multiple Tissue Northern blots (CLONTECH) were hybridized with a ^{32}P-labeled 1.1-kb human DGAT fragment from the human EST. For mouse tissues, total RNA was prepared with Trizol reagent (Life Technologies), and samples (10 μg) were analyzed by Northern blot with a ^{32}P-labeled, 1-kb mouse DGAT fragment from the mouse EST. Blots were stripped and sequentially reprobed for glyceraldehyde-3-phosphate dehydrogenase and 28S RNA (20). Bands in autoradiograms from the 3T3-L1 experiments were quantified with a PhosphorImager (Fuji Medical Systems, Stamford, CT).

NIH 3T3-L1 Differentiation. NIH 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. 3T3-L1 cell differentiation into adipocytes was induced by incubating confluent monolayers of cells in serum-containing medium supplemented with 10^{-5} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/ml insulin (21).

Gene Mapping. Primers derived from the human EST sequences were used to identify genomic clones in an arrayed bacteria artificial chromosome (BAC) library according to the manufacturer's protocol (Research Genetics, Huntsville, AL). The BAC clone was mapped to chromosome 8qter by fluorescent *in situ* hybridization (22). The clone (RMC08P051) may be requested from the website http://rmc-www.lbl.gov. Linkage analysis for mouse gene mapping was performed with a panel of 67 progeny derived from an interspecific backcross [(C57BL/6J× *Mus spretus*)F_1 × C57BL/6J] (23). This backcross panel has been typed for more than 400 loci throughout the genome (24). Briefly, parental strain DNAs were screened for restriction fragment-length variants by restriction enzyme digestion and hybridization with a radiolabeled, 1-kb mouse DGAT cDNA fragment as described (23). Filters were washed in 1.0× SSC/0.1% SDS at 50°C for 20 min. Autoradiograms were exposed for 3 days at −70°C. Linkage to previously typed chromosomal markers was detected by using MAP MANAGER version 2.6.5, and loci were ordered by minimizing the number of recombination events between DGAT and the markers (25).

RESULTS

Through homology searches of the EST databases using coding sequences from ACAT, we identified an EST clone that shared homology with sequences in the ACAT C terminus. The translation of a full-length cDNA for this EST predicts an ORF encoding a 498-aa protein that is ≈20% identical to mouse ACAT (Fig. 2*A*), with the most highly conserved regions in the C terminus. The predicted protein sequence contains a potential N-linked glycosylation site and a putative tyrosine phosphorylation site. A serine residue found in ACAT that is necessary for enzyme activity (26) appears to be conserved. The protein has multiple hydrophobic domains and 6–12 possible transmembrane domains (Fig. 2*B*). Analysis by a transmembrane region prediction program (http://ulrec3.unil.ch/software/TMPRED_form.html) favors nine transmembrane domains (amino acids 96–114, 140–157, 174–198, 200–218, 293–311, 337–360, 412–434, 436–456, and 461–484).

Given the 20% sequence identity to ACAT, experiments were designed to test whether this cDNA encoded an enzyme that catalyzed cholesterol esterification. FLAG epitope tagged (at the N terminus) or untagged versions of the cDNA were expressed in H5 insect cells by using a baculovirus expression system. Cells infected with the virus containing this cDNA

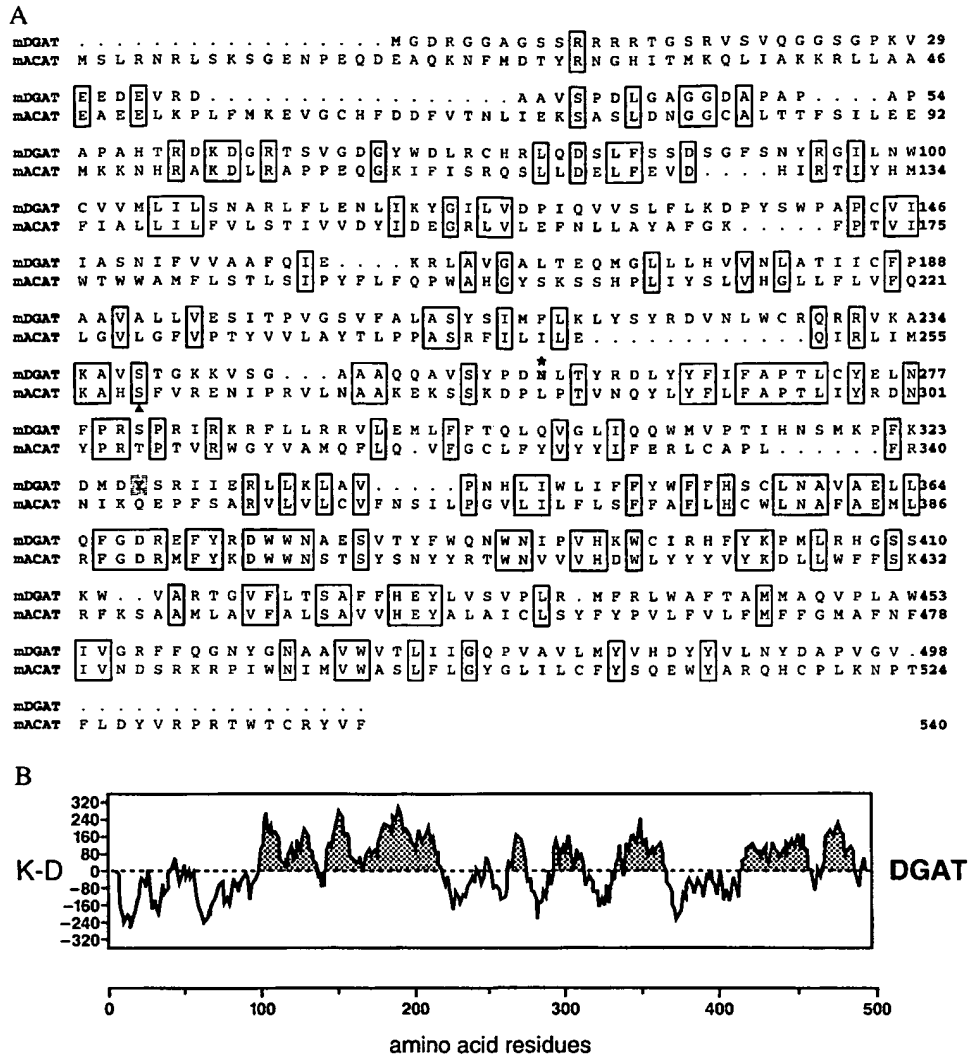

FIG. 2. The mouse DGAT protein. (*A*) Predicted amino acid sequence of the mouse DGAT cDNA. The predicted amino acid sequence of mouse DGAT (mDGAT) is shown in alignment with mouse ACAT (mACAT) (32). The two sequences are ≈20% identical (identical residues are boxed). A potential N-linked glycosylation site (asterisk) and tyrosine phosphorylation site (shaded) are indicated. A serine residue in ACAT known to be necessary for catalytic function is also indicated (triangle). (*B*) Hydrophobicity plot of DGAT as assessed by Kyte–Doolittle (K–D) analysis (33). Hydrophobic regions are shaded.

expressed an ≈47-kDa protein at high levels in the membrane fraction (Fig. 3*A*) but lacked detectable cholesterol esterification activity as compared with ACAT virus-infected cells (Fig. 3*B*). However, further analysis of TLC plates from these assays revealed that membranes from these cells had significantly increased triacylglycerol mass (as assessed by I_2 visualization) (data not shown) and incorporated significantly more [^{14}C]-oleoyl CoA into triacylglycerols than did membranes from wild-type virus-infected cells (197 vs. 55 pmol/mg protein per min). These data suggested that the identified cDNA encoded a DGAT.

DGAT activity was measured in membranes from H5 insect cells expressing the putative DGAT cDNA and found to be more than 5-fold higher than in membranes from wild-type virus-infected cells (Fig. 3*B*). The DGAT activity level increased proportionately with the amount of FLAG-tagged protein expressed in membranes isolated from cells harvested at different time points after infection (Fig. 3*C*). DGAT activity levels in membranes from cells expressing the cDNA were similar regardless of whether [^{14}C]DAG or [^{14}C]oleoyl CoA was used as the labeled substrate (Fig. 3*D*). In the absence of added oleoyl CoA, [^{14}C]DAG was not incorporated into triacylglycerols. In addition, [^{3}H]oleic acid was not incorporated into triacylglycerols in membranes from DGAT virus-infected cells (7 ± 6 vs. 49 ± 47 pmol triacylglycerol/mg protein per min for wild type, $n = 3$), establishing the requirement for a fatty acyl CoA. Triacylglycerol mass was increased more than 10-fold in membranes from DGAT virus-infected cells compared with membranes from wild-type virus-infected cells (11 ± 7 vs. 1 ± 0.5 pg/μg membrane protein, $P = 0.04$, $n = 5$). No change in relative DAG mass was observed (0.33 ± 0.05 vs. 0.34 ± 0.12 units for DGAT and wild type, respectively). We also tested a variety of other possible acyl acceptors, including 25-hydroxy-, 26-hydroxy-, 7α-hydroxy- or 7β-hydroxycholesterols, 7-ketocholesterol, vitamins D2 and D3, ethanol, β-sitosterol, lanosterol, and ergosterol (shown in Fig. 3*E*), and vitamin E, retinol, and dehydroepiandrosterone (data not shown), as substrates for the expressed DGAT enzyme. Although [^{14}C]oleoyl CoA was consistently incorporated into triacylglycerols (by using the endogenous diacylglycerol as the acyl acceptor), it was not incorporated into esters for any other substrate tested, as assessed by autoradiography of TLC plates used to analyze reaction products (Fig. 3*E*).

DGAT mRNA expression was examined in a cultured cell model of adipocyte differentiation and in mammalian tissues. The mRNA increased markedly (≈8-fold) in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation

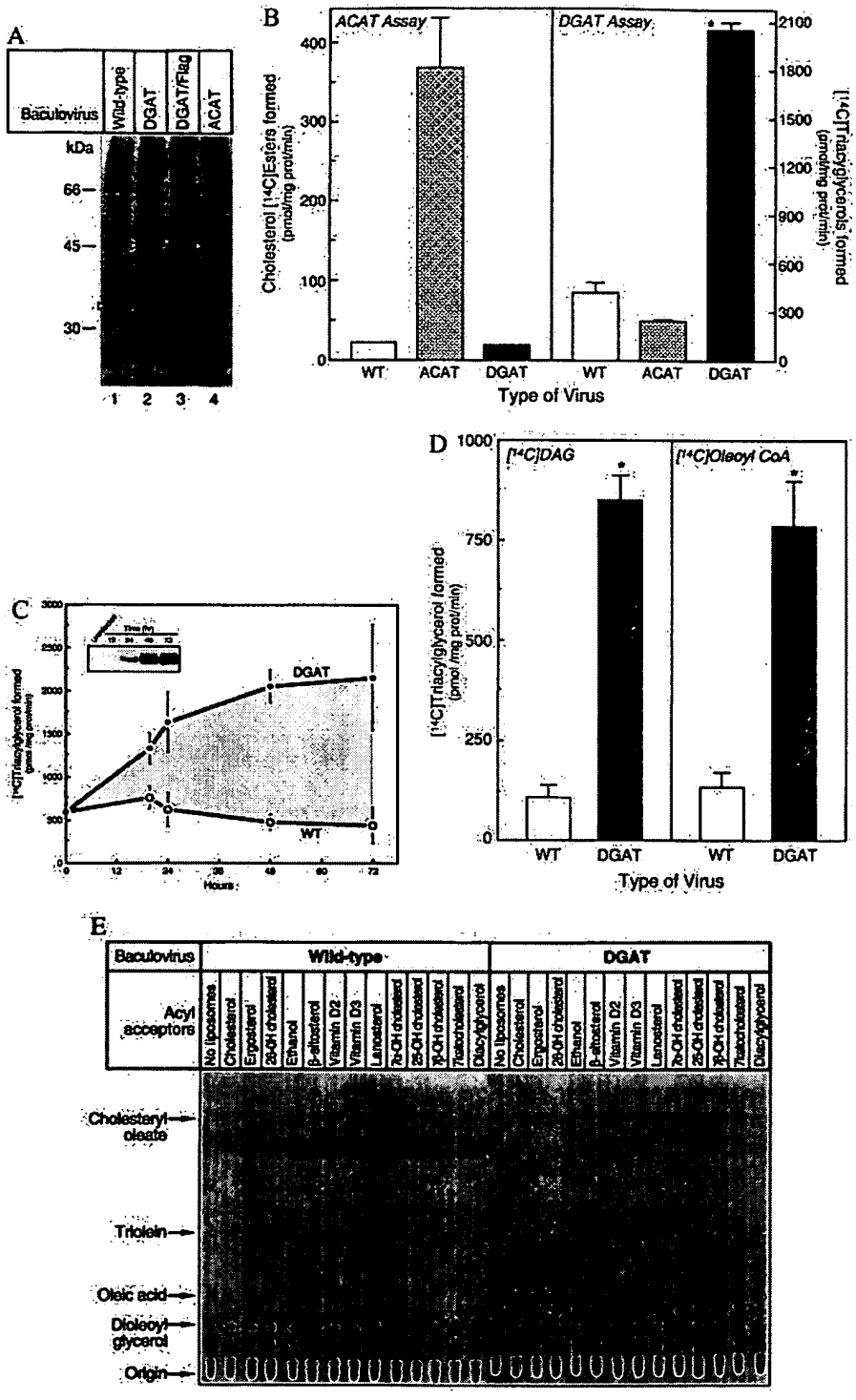

FIG. 3. Enzymatic activities in insect cell membranes expressing DGAT. Cells were infected with wild-type baculovirus (WT), mouse ACAT, or mouse DGAT recombinant baculoviruses, and membranes were assayed for enzymatic activity. (*A*) Metabolic labeling. Cell proteins, 48 h after infection, were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and whole-cell lysates were analyzed by SDS/PAGE and autoradiography. The expression of viral polyhedrin protein (lane 1), mouse DGAT (lane 2), FLAG-tagged mouse DGAT (lane 3), and mouse ACAT (lane 4) is indicated (triangles). (*B*) ACAT and DGAT activities. Data represent the mean (±SE) of five experiments. *, $P < 0.001$. vs. WT. (*C*) Time course of DGAT virus infection. Insect cell membranes were isolated at the indicated times after infection. Expression of the FLAG-tagged DGAT was detected by immunoblotting with an anti-FLAG antibody (*Inset*), and DGAT activity was measured. The doublet band observed in this experiment was not routinely observed, and its significance is unknown. Data represent the mean (±SE) of three experiments. (*D*) Comparison of the rate of triacylglycerol synthesis with either DAG or oleoyl CoA as the radiolabeled substrate. Assays contained the same amounts of oleoyl CoA (5 nmol) and DAG (2.5 μg) in all cases. The specific activity for DGAT virus-infected cells is less than that observed in *A* because of the reduction in DAG substrate concentration (i.e., this experiment was not performed at apparent V_{MAX}). Data are the mean (±SE) of five experiments. *, $P < 0.001$ vs. WT. (*E*) Acyl acceptor specificity of DGAT. Reaction products from wild-type or DGAT virus-infected membranes assayed with [^{14}C]oleoyl CoA and various acyl acceptor substrates were analyzed by TLC. Note that [^{14}C]oleoyl CoA is incorporated specifically into triacylglycerols for all reactions containing membranes expressing DGAT. Hydrolysis of the labeled oleoyl CoA to oleic acid (as shown in this experiment) was observed in some, but not all, preparation of membranes expressing DGAT; this finding was associated with membranes expressing the highest levels of DGAT activity.

into adipocytes (Fig. 4*A* and *B*). DGAT mRNA was expressed in every human (Fig. 4*C*) and mouse (data not shown) tissue examined, with the highest expression levels in the small intestine. In addition, expression was detected in mouse adipose tissue (Fig. 4*D*). In the human Northern blots, additional hybridization signals were observed at ≈2.2 kb and ≈4.0 kb. The significance of these bands is currently unknown.

The human DGAT gene was mapped to human chromosome 8qter by fluorescent *in situ* hybridization. By using an interspecific cross, we mapped the mouse homolog for the DGAT gene (*Dgat*) to a region of chromosome 15 that exhibits homology with human chromosome 8 (Fig. 5). This region of mouse chromosome 15 has exhibited linkage with levels of plasma triacylglycerol-rich lipoproteins in several genetic crosses (24, 27, 28). In each case, the strongest linkage was observed with levels of plasma very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, but there was also evidence of linkage with levels of plasma triacylglycerols. For example, in a cross between strains MRL and BALB/c, marker D15Mit17 exhibited a lod score of 6.7 for VLDL and LDL cholesterol and 2.7 for triacylglycerols (24).

DISCUSSION

DGAT is a microsomal enzyme that plays a central role in the biosynthesis of cellular triacylglycerols. DGAT has never been purified to homogeneity, nor has its gene been cloned. In this study, we identified a cDNA encoding a protein that possesses DGAT activity. The identification of this cDNA provides a molecular probe for studying the role of DGAT in biology.

Several findings support the hypothesis that the cDNA we identified encodes a DGAT. First, DGAT activity was more than 5-fold higher in membranes expressing the cDNA than in membranes from wild-type virus-infected cells. Although insect cells, like all eukaryotic cells, synthesize triacylglycerols (2), the ability to express large amounts of the DGAT protein enabled us to detect DGAT-specific activity at high levels, similar to those found in mammalian tissues (14) and considerably above background levels in insect cells. The acyltransferase activity depended on the presence of a fatty acyl CoA substrate and was specific for DAG; there was no activity with cholesterol or a variety of other acyl acceptor substrates. Second, its mRNA expression increased markedly in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation into adipocytes, a process known to be associated with increases in DGAT activity (29) and triacylglycerol mass accumulation (30). Third, mRNA expression was detected in every mammalian tissue examined, as expected because of the central role of DGAT in cellular glycerolipid metabolism. The highest expression levels were found in the small intestine, consistent with a proposed role for DGAT in intestinal fat

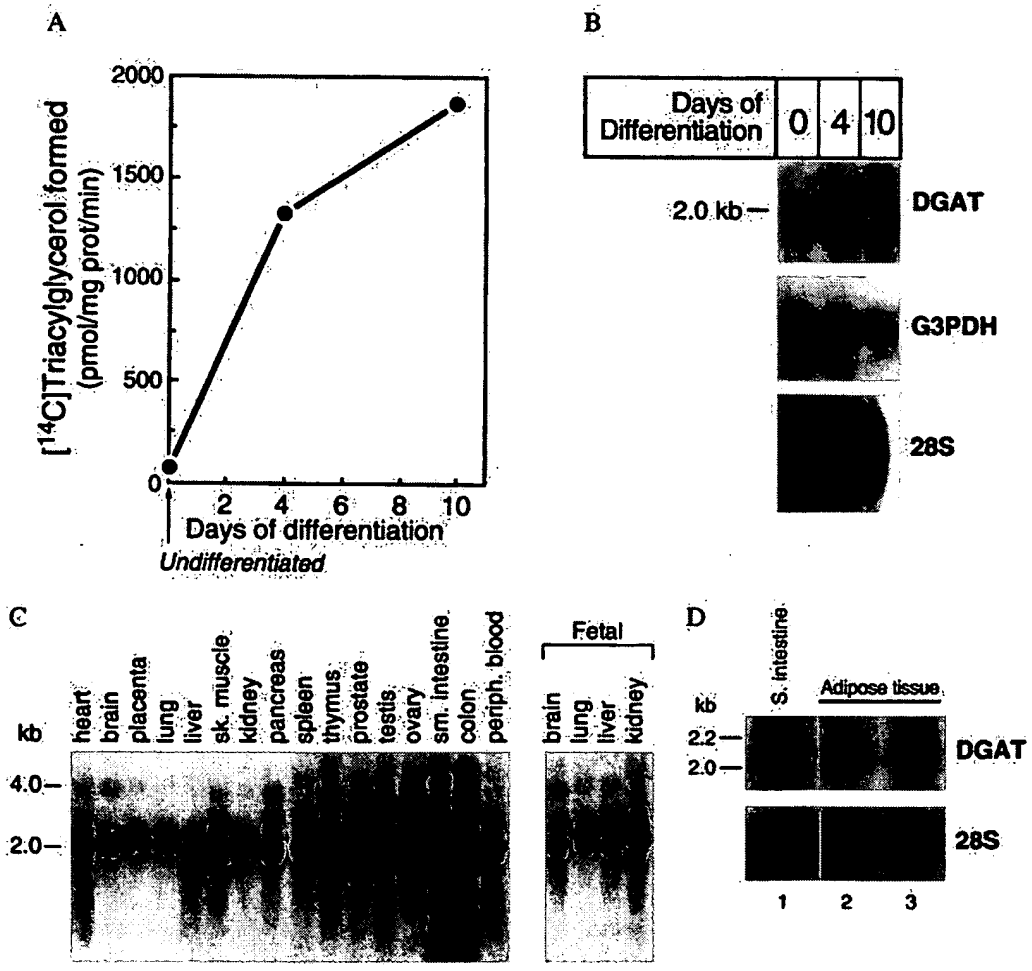

FIG. 4. Analysis of DGAT mRNA expression. (*A*) DGAT activity and (*B*) DGAT mRNA expression during differentiation of 3T3-L1 cells into adipocytes. Mouse 3T3-L1 adipocyte differentiation was induced, and RNA and membranes were isolated from undifferentiated cells or cells harvested 4 and 10 days later. Results are shown for DGAT and two controls for RNA loading [glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and 28S RNA]. Quantitation of DGAT mRNA in triplicate samples, by PhosphorImager analysis and correction for loading relative to 28S RNA (20) as an internal standard, demonstrated that DGAT levels were increased 5-fold by day 4 and 8-fold by day 10 of differentiation. The experiment was repeated three times with similar results. (*C*) DGAT expression in human tissues as assessed by Northern blot analysis. (*D*) DGAT expression in mouse small intestine and adipose tissue from two mice (lanes 2 and 3). The membrane was stripped and reprobed for 28S RNA (20).

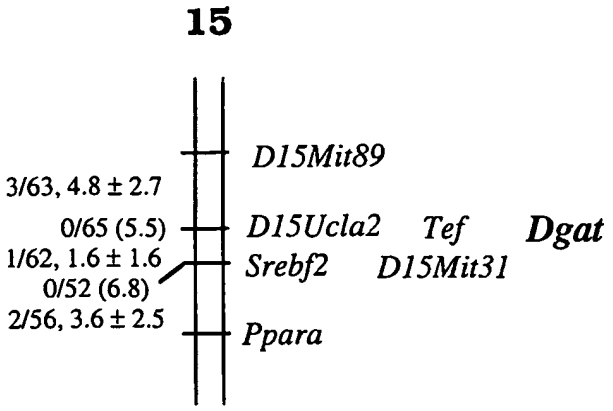

FIG. 5. Mouse chromosomal location of DGAT gene. Genetic mapping of the *Dgat* to mouse chromosome 15 was performed by linkage analysis performed with a panel of 67 progeny from an interspecific backcross [(C57BL/6J × *Mus spretus*)F_1 × C57BL/6J] (23). A segment of the chromosome is drawn with the centromere toward the top. The ratios of the number of recombinants to the total number of informative mice and the recombination frequencies ±SE (in cM) for each pair of loci are indicated. For pairs of loci that cosegregate, the upper 95% confidence interval is shown in parentheses. No recombination was observed between *Dgat*, D15Ucla2, and *Tef* (0/65 mice). The DGAT gene has been assigned the name *Dgat*. The data have been deposited in the Mouse Genome Database under accession number MGD-J:44983.

absorption (3, 31). In addition, the mRNA was expressed in adipose tissue, which is known to have a high level of DGAT activity (6). Interestingly, mRNA expression was relatively low in the livers of humans (Fig. 4*C*) and mice (data not shown), despite the fact that significant DGAT activity is present in the liver (14). The significance of this finding is currently unknown. One possibility is that, although the expression levels are low, the mRNA encodes a very stable protein; alternatively, it is possible that the liver expresses a second DGAT. As a final piece of evidence confirming the identity of this cDNA, we have disrupted the mouse DGAT gene in embryonic stem cells and achieved germ-line transmission of this mutation. Preliminary results indicate that DGAT activity in membranes from embryonic fibroblasts homozygous for the knockout mutation is reduced to ≈5% or less than that in wild-type fibroblast membranes (S.J.S., S.C., and R.V.F., unpublished observations).

The identification of a DGAT cDNA has significant implications for understanding the regulation of the triacylglycerol biosynthetic pathway and intracellular lipid metabolism. DGAT molecular probes will facilitate *in vivo* studies of the role(s) of DGAT and its regulation in a number of physiologic processes, such as intestinal fat absorption, lipoprotein synthesis and secretion, lactation, and adipose tissue formation. Whether DGAT regulation participates in modulating levels of cellular DAG involved in signaling also can be assessed. Ultimately, understanding DGAT at a molecular level may uncover potential approaches for treating hypertriglyceridemia or obesity in humans.

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Characterization of Two Human Genes Encoding Acyl Coenzyme A: Cholesterol Acyltransferase-related Enzymes*

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The enzyme acyl coenzyme A:cholesterol acyltransferase 1 (ACAT1) mediates sterol esterification, a crucial component of intracellular lipid homeostasis. Two enzymes catalyze this activity in *Saccharomyces cerevisiae* (yeast), and several lines of evidence suggest multigene families may also exist in mammals. Using the human ACAT1 sequence to screen data bases of expressed sequence tags, we identified two novel and distinct partial human cDNAs. Full-length cDNA clones for these ACAT related gene products (ARGP) 1 and 2 were isolated from a hepatocyte (HepG2) cDNA library. ARGP1 was expressed in numerous human adult tissues and tissue culture cell lines, whereas expression of ARGP2 was more restricted. *In vitro* microsomal assays in a yeast strain deleted for both esterification genes and completely deficient in sterol esterification indicated that ARGP2 esterified cholesterol while ARGP1 did not. In contrast to ACAT1 and similar to liver esterification, the activity of ARGP2 was relatively resistant to a histidine active site modifier. ARGP2 is therefore a tissue-specific sterol esterification enzyme which we thus designated ACAT2. We speculate that ARGP1 participates in the coenzyme A-dependent acylation of substrate(s) other than cholesterol. Consistent with this hypothesis, ARGP1, unlike any other member of this multigene family, possesses a predicted diacylglycerol binding motif suggesting that it may perform the last acylation in triglyceride biosynthesis.

The intracellular formation of sterol esters from fatty acid and sterol is mediated by acyl-CoA:cholesterol acyltransferase (ACAT).[1] The pathological accumulation of cholesterol esters in atherosclerotic lesions has lead to intense pursuit of ACAT inhibitors as pharmacological agents. Microsomal ACAT preparations from various tissues display differential sensitivities to some of these agents (1) including histidine modifiers (2). This suggests that more than one protein mediates the esterification reaction, such as occurs in yeast (reviewed in Ref. 3). *Saccharomyces cerevisiae* (budding yeast) has two ACAT related enzymes, Are1 and Are2, which are derived from separate genes and have been shown to independently esterify sterols (4, 5). In terms of contribution to the sterol ester mass of the cell, Are1 is the minor isoform relative to Are2. These genes were identified based on sequence conservation to a human gene, ACAT1, which encodes an ACAT enzyme with homologs in many mammalian species (6, 7). The human ACAT1 gene encodes a 550-amino acid polypeptide and is expressed in most tissues, predominantly placenta, lung, kidney, and pancreas (6). ACAT1 has been predicted to have two transmembrane domains (6) and has been immunolocalized to the endoplasmic reticulum (8, 9). When murine ACAT1 was disrupted in induced mutant mice, homozygotes for the deletion were found to essentially lack ACAT activity in embryonic fibroblasts and have negligible amounts of cholesterol ester in the adrenal cortex and peritoneal macrophages (10). However, cholesterol ester accumulation was normal in hepatocytes while dietary cholesterol absorption, an indirect marker for intestinal cholesterol esterification, was indistinguishable from control littermates. This is consistent with the concept of a multigene family for this activity.

ACAT isoenzymes may be required to perform the variety of physiological roles mediated by cholesterol esterification. Increases in cellular free cholesterol above certain levels are cytotoxic and are ameliorated by cholesterol ester formation (11). In hepatocytes, the bulk of cholesterol secreted in very low density lipoprotein is esterified intracellularly and determines apolipoprotein B secretion rates (12–14). Cholesterol esterification in the enterocyte may be necessary for cholesterol absorption from the lumen and secretion in chylomicrons into the lymph (15). The formation of cholesterol ester stores could also provide a readily available substrate for steroid hormone synthesis in steroidogenic tissues (16, 17). It is likely that different ACAT isozymes mediate each of these processes, and the data presented here support that hypothesis.

We reasoned that additional human ACAT proteins would have sequence similarity to regions conserved between human ACAT1 and yeast Are1 and Are2. (4). Accordingly, an ACAT consensus sequence was used to screen the data base of expressed sequence tags (dbEST). Several cDNA entries were identified which were transcribed from two independent human genes. This study is a description of the isolation of full-length cDNA clones for two ACAT-related gene products (ARGP1 and ARGP2), examination of their pattern of tissue

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF059202 and AF059203.

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[1] The abbreviations used are: ACAT, acyl coenzyme A:cholesterol acyltransferase; ARGP, ACAT related gene product; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); RT, reverse transcriptase; DEPC, diethylpyrocarbonate.

expression, and assays of enzymatic activity. We show that ARGP2 can catalyze the formation of sterol ester from cholesterol and oleoyl-CoA, leading us to rename this gene, ACAT2. By contrast, ARGP1 did not detectably esterify cholesterol and we propose that it performs acyl-CoA-dependent acylation of other molecules, such as diacylglycerol.

EXPERIMENTAL PROCEDURES

General—Molecular biology techniques were performed by conventional protocols (18, 19) and DNA modifying reagents were purchased from Life Technologies, Inc., New England Biolabs, or Promega as indicated. The Prime It random priming probe synthesis kit was obtained from Stratagene. The DIG Genius probe synthesis kit and CSPD were supplied by Boerhinger Mannheim. Radioactive reagents ([^{14}C]oleoyl-CoA and [^{32}P]dCTP) were purchased from NEN Life Science Products Inc. Ethidium bromide-stained agarose gels were visualized by the Kodak Digital Science 1D system. Automated DNA sequencing was performed at the Columbia University Cancer Center sequencing facility, and oligonucleotides were synthesized by Genset. DNA and amino acid sequence analysis and comparisons were performed using DNAStrider (20), PILEUP, and GAP programs (GCG Inc. (21)), Prosite (22), and Identify (Ref. 23, website http://dna.stanford.edu/identify/). Yeast media components were prepared as described (18).

Screening the dbEST—A 30-amino acid ACAT concensus peptide sequence (FAEMLRFGDRMFYKDWWNSTSYSNYYRTWN) was used as the query in a tblastn (which compares a protein sequence against a nucleotide sequence data base translated in all reading frames (24, 25)) search of the data base of expressed sequence tags at NCBLI (dbEST). Three clones, H24971, R07932, and R99213, derived from a common gene (named ACAT related gene product 1, ARGP1), were identified ($p < 10^{-4}$). The entire human ACAT1 protein was then used in an identical search. In addition to clones of ACAT1 and ARGP1, two entries, R10272 and W76421, with significant similarity were identified ($p < 10^{-5}$). They were derived from a gene we named ARGP2. Rescreening the dbEST with these clones identified two more ARGP2 entries. *Escherichia coli* clones with the largest inserts corresponding to these sequences were obtained from the I.M.A.G.E. consortium and resequenced with T3, T7, or gene specific primers.

5′ Rapid Amplification of cDNA Ends (RACE) of ARGP1—Oligo(dT) primed, double stranded cDNA was reverse transcribed from human, ileal, poly(A)$^+$ mRNA, kindly provided by Dr. Paul Dawson, and ligated to adapters using a commercially available kit (CLONTECH, Palo Alto, CA). Touchdown PCR (26) was performed for 35 cycles with a forward primer complementary to the adapter (AP1, 5′-CCATCCTAATACGACTCACTATAGGGC) and a reverse primer (End4A, 5′-CCACCTGGAGCTGGGTGAAGAAC) complementary to the ARGP1 dbEST clone Z43867. The PCR mixture included 200 nM each oligo, 200 μM dNTPs, 1.75 mM $MgCl_2$, 2.5 units of *Taq*, and the cDNA diluted 1:500. The 700-bp reaction product was gel isolated, ligated into YEp352 with a T overhang generated by *Taq* polymerase, and sequenced.

5′ RACE of ARGP2—A human, fetal (20 weeks post-conception) liver/spleen, oligo(dT)-primed, cDNA library in the vector pT7T3D was kindly provided by Dr. Bento Soares. PCR was performed with the cDNA, a forward primer (M13 reverse, 5′-TGAGCGGATAACAATTTCACACAGG) complementary to the vector and a reverse primer (203, 5′-CCCCATGCTGAGGTCTGTGATCAG), complementary to the ARGP2 dbEST clone R10272, using the above conditions. The 800-bp reaction product was gel isolated, ligated into pBS:SK (Stratagene) with a T overhang generated by *Taq* polymerase, and sequenced.

Hybridization Screening of a HepG2 cDNA Library—A yeast expression library of HepG2 cDNA (size selected for inserts greater than 2.0 kb in pAB23BXN, commercially available from Austral Biologicals, San Ramon, CA), was propagated in the *E. coli* strain MC1061 and plated onto 135-mm LB + ampicillin (50 μg/ml) plates at an approximate density of 5000 colonies per plate. Membrane (Hybond-N, Amersham) replicas of the plates were probed by hybridization with a digoxigenin-labeled probe specific for ARGP1 (synthesized using a 420-bp *Not*I, *Pst*I digestion product of the 5′ RACE product) or ARGP2 (synthesized using the 5′ RACE product) in 5 × SSC, 0.05% SDS, 0.1% *N*-laurolysarcosine, 0.1 mg/ml salmon sperm DNA, and 2% (w/v) blocking reagent (Boerhinger Mannheim) at 65 °C for 14–18 h. The membranes were washed in 0.2 × SSC, 0.1% SDS at 60 °C for 80 min, incubated with an anti-digoxigenin antibody (1:10,000), washed in Tris-buffered saline, incubated with the peroxidase substrate CSPD (Boerhinger Mannheim), and detected by enhanced chemiluminescence (ECL). For ARGP1, 4 single positive clones were isolated after screening ~20,000 clones. For ARGP2, 4 single positive clones were isolated after screening ~30,000 clones. The longest clones for each were sequenced multiple times on both strands using vector and gene-specific oligonucleotides.

Tissue Culture—Cultured human Caco2, HeLa, HepG2, and THP1 cell lines were donated by Dr. R. J. Deckelbaum and originally obtained from the ATCC. HepG2, HeLa, and Caco2 cells were maintained as cell monolayers in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) + 10% fetal bovine serum (HyClone) in 5% CO_2. THP1 monocyte cells were maintained in suspension in RPMI (Life Technologies, Inc.) + 10% fetal bovine serum in 5% CO_2. Differentiation of THP1 cells was stimulated with 150 ng/ml tetramyristate phorbol ester and 140 μM β-mercaptoethanol. Whole cell RNA was isolated from confluent monolayer cultures or pelleted THP1 cells using TRIzol (Life Technologies, Inc.) extraction. The Caco2 cells had been confluent for approximately 21 days.

Human Adult and Fetal Multi-tissue Northern Blot Analysis—Commercially obtained multi-tissue Northern blot (CLONTECH) contained 2 μg of poly(A)$^+$ RNA from human adult or fetal (18–24 weeks postconception) tissues originally resolved on a 1.2% agarose, formaldehyde gel. The adult tissue membrane was hybridized with a random-hexamer primed, [^{32}P]dCTP-labeled probe, generated using the insert of the ARGP1 dbEST clone R99213, in ExpressHyb buffer (CLONTECH) for 1 h at 68 °C. The membrane was washed in 0.1 × SSC, 0.5% SDS at 50 °C. After stripping the membrane was probed with ARGP2 (dbEST clone 10272 insert and the ARGP2 5′ RACE product) using the conditions above. The fetal tissue Northern blot was hybridized with the same ARGP2 probe.

Reverse Transcription PCR—Human cDNA obtained as part of a Quick Screen cDNA Panel of Human tissues (CLONTECH) or reverse transcribed (Life Technologies, Inc. kit) from human ileal poly(A)$^+$ mRNA was used as the template in a PCR reaction with primers specific for ARGP1 (106, GGCATCCTGAACTGGTGTGTGGTG; 110, AGCTGGCATCAGACTGTGTCTGG), ARGP2 (202, GAGTTCCCCCACATTCATCAAATCC; 206, CATGCTGCTGCTCATCTTCTTTGCA), or β-actin (Act1, GAGCTGCCTGACGGCCAGGTC; Act2, CACATCTGCTGGAAGGTGGACAG). The PCR mixture included 1.5 mM $MgCl_2$, 200 μM dNTPs, 400 nM of each primer, and 2 units of *Taq* (Life Technologies, Inc.). Following 35 cycles (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min), the products were resolved on a 1% agarose, 0.5 μg/ml ethidium bromide gel. For RNA prepared from human cultured cells, first strand cDNA synthesis was performed, with and without reverse transcriptase (SuperscriptII, Life Technologies, Inc.) using 4 μg of whole cell RNA. A fraction of each reaction (10%) was the template in a PCR reaction (30 cycles of 94 °C, 30 s; 68 °C, 2 min) with primers specific for ARGP1 (103, GCTTCATGGAGTTCTGGATGGTGG; 106, GGCATCCTGAACTGGTGTGTGGTG), ARGP2 (201, GACACCTCGATCTTGGTCCTGCC; 202, as above) or human ACAT1 (ACATa, CGGAATATCAAACAGGAGCCCTTC; ACATb, CATTCCAAAGAACATGAAGATGCACG).

In Vitro Assay of ACAT Activity in Yeast Microsomes—The cDNA inserts of the longest ARGP1 and ARGP2 HepG2 library clones were removed by *Not*I, *Eco*RI digestion and ligated into the yeast expression vector pRS426GP which utilizes the galactose inducible GAL1/GAL10 promoter. A cDNA corresponding to the coding region of human ACAT1 flanked by 5 bp of 5′-untranslated region and 1 bp of 3′-untranslated region, in pRS426GP was described previously (27). Yeast strain, SCY059 (MAT*a*, *ade2-1, can1-1, trp1-1, ura3-1, his3-11, 15, leu2-3, 112, met14Δ14HpaI-SalI, are1ΔNA::HIS3, are2Δ::LEU2*) with deletions in *ARE1* and *ARE2*, the yeast homologs of human ACAT1 (4), was transformed with the above constructs or pRS426GP using lithium acetate and nucleic acid prototrophy selection (28). Expression of the constructs was verified by RT-PCR analysis of RNA isolated from the transformed cells. Culturing of the transformed yeast, induction of expression, microsome isolation, and sterol esterification assays were as described previously (27). In those experiments involving diethylpyrocarbonate (DEPC) to modify histidine residues, a preincubation with 100 μM DEPC was performed as described (2).

RESULTS

Isolation of Full-length cDNA Clones for Two ACAT Related Human Genes—A comparison of the human ACAT1 protein and the two yeast ACAT orthologs (Are1, Are2) identified a highly conserved (70% identical) region of 30 amino acids (ACAT1 amino acids 391–420) near the carboxyl terminus. This peptide was used to screen the data base of expressed sequence tags (dbEST). The search identified several human cDNAs, the longest being 890 bp (GenBank accession number H45923), derived from a common gene we call the ARGP1. To

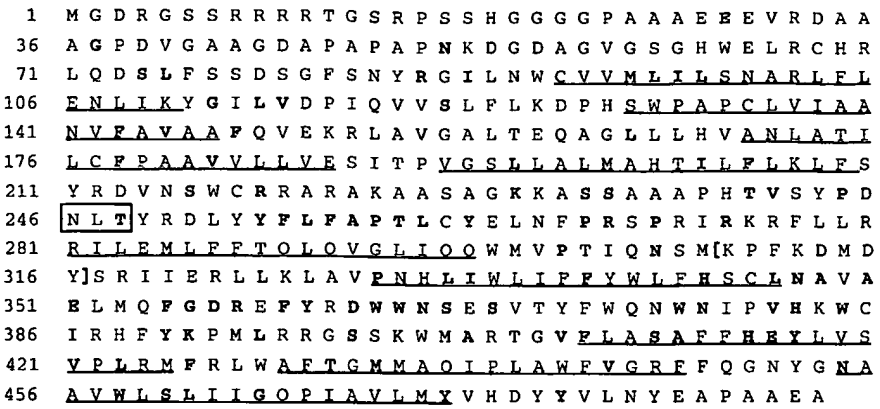

FIG. 1. **ARGP1 predicted peptide sequence.** A 1976-bp ARGP1 cDNA clone was identified by colony hybridization screening of a HepG2 cDNA library. Translation of this clone predicts the 488-amino acid peptide shown. The residues in *bold* are conserved with human ACAT1. The *underlined* portions are predicted transmembrane domains, a potential *N*-linked glycosylation site is *boxed*, and a putative tyrosine phosphorylation motif is in *brackets*. The sequence has been deposited at GenBank, accession number AF059202.

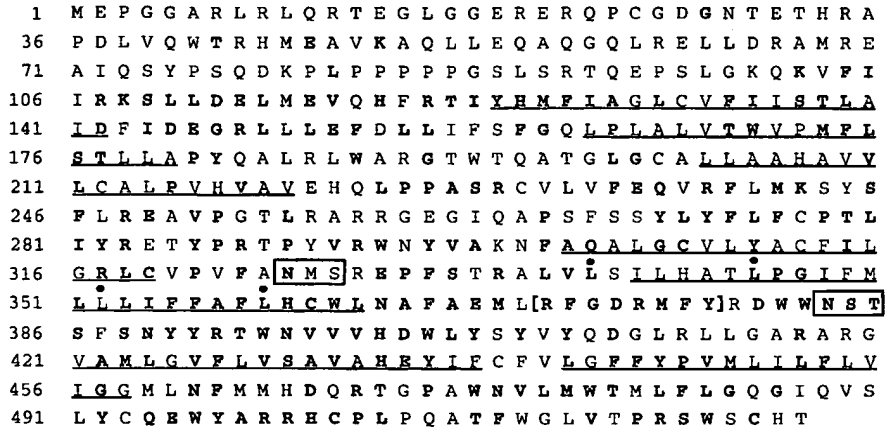

FIG. 2. **ARGP2 predicted peptide sequence.** A 2040-bp ARGP2 cDNA isolated by screening a HepG2 cDNA predicts the 522-amino acid polypeptide shown. The residues in *bold* are conserved with human ACAT1. The *underlined* portions are predicted transmembrane domains, two potential *N*-linked glycosylation sites are *boxed*, a putative tyrosine phosphorylation motif is in *brackets*, and the *circles* mark the leucine zipper heptad motif. The sequence has been deposited at GenBank, accession number AF059203.

date, 26 clones for human ARGP1 are present in the dbEST from fetal liver/spleen, infant brain, breast, cerebellum, hippocampus, kidney, placenta, testis, ovary tumor, colon tumor, and lung tumor libraries, suggesting ubiquitous and abundant expression. In addition, ARGP1 is also represented as several murine entries (*e.g.* GenBank accession number C75990). The dbEST was then searched using the entire ACAT1 protein sequence. Four human cDNAs, distinct from ARGP1 cDNA clones, were identified in fetal liver/spleen and fetal heart libraries and are derived from a common gene we call ARGP2. The longest entry was 600 bp (GenBank accession number R10272). To date these are the only dbEST entries for human ARGP2, although several murine entries have been identified (*e.g.* GenBank accession number AA410072).

Northern blot analysis of human tissues (Figs. 3 and 4) showed that the initial dbEST clones for ARGP1 and ARGP2 were truncated, relative to the observed transcripts, by approximately 1000 and 1400 bp, respectively. To isolate full-length cDNA clones, 5′ RACE was performed using cDNA synthesized from human liver (ARGP1) or ileal (ARGP2) mRNA but yielded only 600 nucleotides of novel sequence for each. The respective 5′ RACE products were then used as probes to screen a size selected (>2.0 kb), HepG2 cDNA library by hybridization. The longest ARGP1 clone contained 1976 nucleotides and a 130-base poly(A)$^+$ tract which agreed with the length of the minimal ARGP1 transcript detected by Northern blot (Fig. 3). HepG2 cells express only the 2.0-kb ARGP1 transcript.[2] A similar approach identified ARGP2 clones, the longest of which contained 2040 bp of sequence with a 50-base poly(A)$^+$ tract in accordance with the observed length of the ARGP2 transcript (Fig. 4).

ARGP1 Predicted Peptide—The longest open reading frame of ARGP1, flanked by a 244 nucleotide 5′-untranslated region and a 265-nucleotide 3′-untranslated region, encodes a 488-amino acid protein (Fig. 1) with a calculated molecular mass of 55,216 daltons. The predicted initiator methionine lies within a consensus for initiation of translation (29) and downstream of an in-frame termination codon. Comparison to ACAT1 revealed 22% amino acid sequence identity (29% similarity) over the entire molecule. The conservation of these molecules is greatest toward the COOH terminus, such that ACAT1 and ARGP1 are 28% identical over the last 250 residues. This pattern of sequence similarity is strikingly similar to that observed from comparison of ACAT1 with the yeast Are1 and Are2 proteins. ARGP1 is predicted to be a membrane bound protein with nine putative transmembrane domains and one *N*-linked glycosylation site. Uniquely, ARGP1 contains a diacylglycerol/phorbol ester binding signature sequence (H.[FWY]..[KR].F..P) at amino acids 382–392 which was originally identified by comparison of protein kinase C isoforms and diacylglycerol kinases (Fig. 7) (36, 37)). This motif is also conserved in the murine homolog of ARGP1 residing at the dbEST (GenBank accession number AA764382).

ARGP2 Predicted Peptide—The longest ARGP2 open reading frame, flanked by a 51-nucleotide 5′-untranslated region and a 420-nucleotide 3′-untranslated region, predicts a 522-amino acid protein with a calculated molecular mass of 59,942 daltons (Fig. 2). The predicted initiator methionine lies within a consensus for initiation of translation (29). Over the entire molecule, the predicted protein is 47% identical (54% similar) to human ACAT1. This conservation is even more pronounced at the COOH-terminal end of the molecules, raising to 63% identity over the last 250 residues. ARGP2 is predicted to be a membrane bound protein with seven putative transmembrane domains and two *N*-linked glycosylation sites. ARGP2 is similar to ACAT1 in that it contains a leucine zipper (338–359) which may mediate multimerization or interaction with other proteins. ARGP2 does not pos-

[2] T. Seo, P. Oelkers, M. Giattina, R. J. Deckelbaum, and S. L. Sturley, manuscript in preparation.

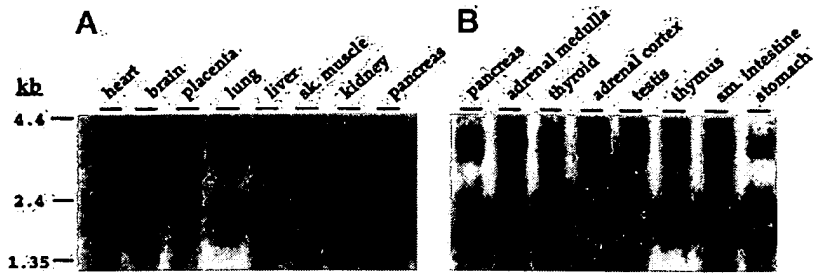

FIG. 3. **Northern blot analysis of ARGP1 expression in human adult tissues.** 2 μg of mRNA from human adult tissues (*panel A*, CLONTECH MTN I; and *panel B*, endocrine system MTN) was hybridized with a [^{32}P]dCTP, random-hexamer labeled, human ARGP1 probe in Express Hyb solution for 1 h at 68 °C. After washing in 0.2 × SSC, 0.1% SDS at 60 °C for 40 min, the membranes were exposed to x-ray film. Molecular weight markers were as supplied by CLONTECH.

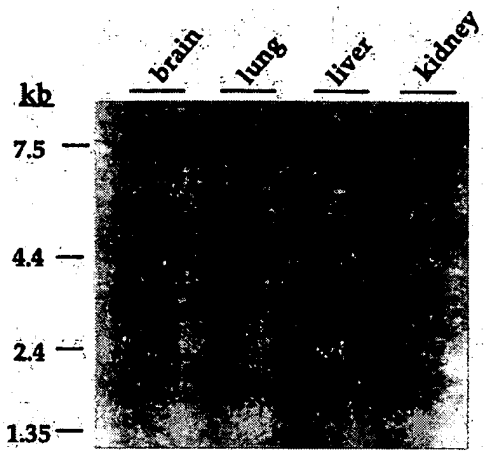

FIG. 4. **Northern blot analysis of ARGP2 expression in human fetal tissues.** 2 μg of mRNA from human fetal tissues (CLONTECH Fetal MTN II) was resolved on a denaturing, 1.2% agarose gel, transferred to a nylon membrane, and hybridized with a [^{32}P]dCTP, random-hexamer labeled, human ARGP2 probe in Express Hyb solution for 1 h at 65 °C. After washing in 0.2 × SSC, 0.1% SDS at 68 °C for 40 min, the membranes were exposed to x-ray film. Molecular weight markers were as supplied by CLONTECH.

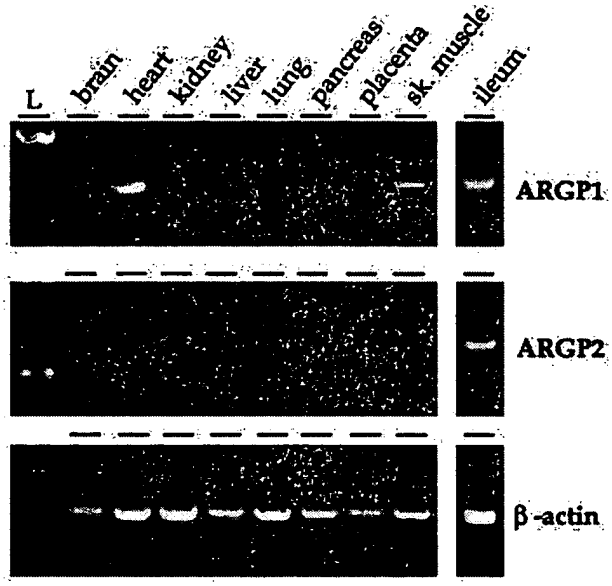

FIG. 5. **Analysis of ARGP1 and ARGP2 expression in adult human tissues using RT-PCR.** PCR was performed as described using a Quick Screen Human cDNA Panel (CLONTECH), or cDNA reverse transcribed from human ileal poly(A)$^+$ mRNA, and primers specific for ARGP1, ARGP2, or β-actin in a standard PCR mixture. The PCR products, predicted to be 921 (ARGP1), 844 (ARGP2), or 835 (β-actin) bp, were resolved on ethidium bromide-stained agarose gels with a 100-bp DNA ladder (*L*; Life Technologies, Inc.).

sess a predicted diacylglycerol/phorbol ester-binding site. A sequenced tag entry (number WI-11660) for ARGP2 localizes to human chromosome 12, further distinguishing it from ACAT1, which is located on chromosome 1 (30).

ARGP1 and ARGP2 Expression in Human Tissues and Tissue Culture Cell Lines—Expression of a second ACAT would be expected in tissues (*e.g.* liver and intestine) which exhibit normal ACAT activity in the induced mutant ACAT1 (*acact*$^-$) mice (10). Expression of ARGP1 and ARGP2 was thus examined by Northern blot of human RNA (Figs. 3 and 4). Hybridization of an ARGP1 cDNA probe to a panel of adult tissue mRNAs detected a transcript in all tissues examined (Fig. 3). However, ARGP1 expression levels varied qualitatively among tissues with moderate expression in thyroid, stomach, heart, skeletal muscle, and liver and high expression in adrenal cortex, adrenal medulla, testes, and small intestine. The presence of a 2.0-kb transcript was invariable among the tissues while a 2.4-kb transcript was observed in about half the tissues, most notably the tissues with high expression. The origin of these two transcripts has not been identified, however, their heterogeneity is unlikely to lie at the 3′ end of the message since all dbEST entries for ARGP1 cDNAs terminate at a similar position. Hybridization of the same membrane, under identical conditions, with an ARGP2 cDNA probe failed to detect a transcript in any tissue (data not shown). Since the four ARGP2 dbEST clones were in human fetal libraries, ARGP2 expression was examined in human fetal tissues by Northern blot (Fig. 4). A 2.2-kb transcript was detected in fetal liver but not in fetal brain, lung, or kidney.

To further examine the expression of ACAT2 in adults, a RT-PCR was performed using cDNA generated from a variety of tissues (Fig. 5). As shown, ARGP2 is expressed in human adult heart, kidney, liver, lung, pancreas, and ileum. The identity of the PCR product was verified by Southern blotting and hybridization with an ARGP2-specific cDNA probe (data not shown). An RT-PCR analysis of ARGP1 on these same samples gave a similar pattern of expression to that determined by the Northern blot in Fig. 3.

ARGP1 and ARGP2 expression in human tissue culture cell lines was also examined by RT-PCR (Fig. 6). ARGP1 was expressed in cell culture models for human endothelial (HeLa), hepatocyte (HepG2), monocyte (undifferentiated THP1), macrophage (differentiated THP1), and intestinal epithelial (Caco2) cells. Expression of ARGP2 was limited to HepG2 and Caco2 cells. This reinforces the concept that ARGP1 is widely expressed while the expression of ARGP2 is more restricted. ACAT1 was expressed in all of these cell lines confirming previous observations (7, 31) (data not shown).

Assay of ACAT Activity in ACAT Negative Yeast Transformed with ARGP1 and ARGP2—The ability of ARGP1 and ARGP2 to esterify sterols was assayed in a sterol esterification deficient yeast strain (SCY059) in which the endogenous *ARE* genes were deleted (27). Microsomes from these yeast, transformed with an expression vector harboring no insert or cDNA inserts for ARGP1, ARGP2, or human ACAT1 were assayed *in vitro* for the incorporation of [^{14}C]oleate into sterol ester. Since we previously demonstrated that cholesterol is the preferred substrate for mammalian ACAT enzymes (27, 32), assays were performed with exogenous cholesterol supplied in Triton WR-1339. As shown in Table I, ARGP2 forms cholesterol ester at a

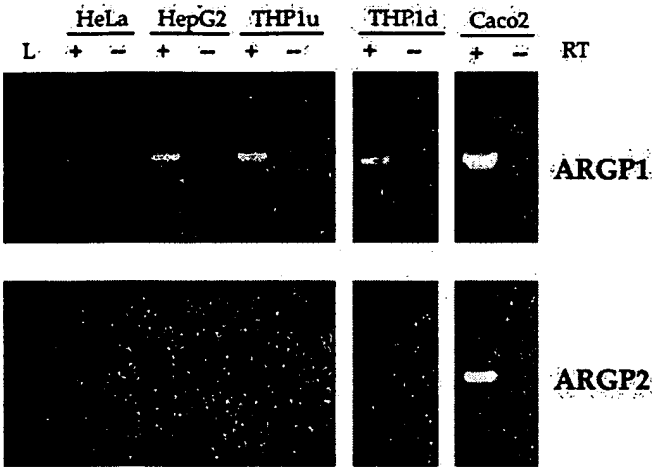

FIG. 6. **Analysis of ARGP1 and ARGP2 expression in tissue culture cells using RT-PCR.** Monolayer cultures of HeLa, HepG2, undifferentiated THP1, and Caco2 tissue culture cells were grown as described under "Experimental Pprocedures." THP1 cells were differentiated into macrophages by the addition of phorbol ester. Total cellular RNA was isolated from the cells and reverse transcribed using oligo(dT) priming in parallel with reactions which lacked RT enzyme. Oligonucleotide pairs complementary to ARGP1 or ARGP2 were included in a PCR using the conditions described in the legend to Fig. 5. The PCR products, predicted to be 667 (ARGP1) and 352 (ARGP2) bp, were resolved on an ethidium bromide-stained agarose gel alongside a 100-bp DNA ladder (*L*; Life Technologies, Inc.).

TABLE I
In vitro analysis of ACAT activity of transformed yeast

S. cerevisiae strain SCY059 (*are1⁻are2⁻*) was transformed with the yeast expression vector pRS426GP harboring either no insert or cDNAs encoding human ACAT1, ARGP1, or ARGP2. Expression was under the control of the inducible *GAL1/10* promoter. Microsomes were isolated from galactose induced yeast cultures and incubated in 0.1 M sodium phosphate, 1 mM glutathione, 20 nM [^{14}C]oleate, with exogenous cholesterol (260 μM in Triton WR-1339) for 3 minutes at 37 °C. The amount of radioactivity incorporated into sterol ester was determined by thin layer chromatography and scintillation counting as described. In those experiments involving DEPC, microsomes were preincubated at room temperature in the presence or absence of 100 μM DEPC for 30 min prior to the ACAT assay. Data are pmol cholesteryl oleate formed per min/mg of protein expressed as mean ± S.E. from at least three different experiments on different preparations or of a representative experiment performed in triplicate.

cDNA Expressed	In vitro microsomal ACAT activity[a]	Active site modification[b]		
		Minus DEPC	Plus DEPC (100 μM)	Degree of inhibition
No insert	2 ± 2			
ACAT1	340 ± 76	340 ± 16	69 ± 4	80%
ARGP1	4 ± 3			
ACAT2	49 ± 15	45 ± 5	30 ± 1	33%

[a] Representative of three different experiments on different preparations.
[b] Representative experiment performed in triplicate.

rate of 49 pmol/min/mg of microsomal protein. This is 24-fold over background and about 15% of the activity detected in microsomes from ACAT1 transformants. We therefore renamed ARGP2 as ACAT2. ARGP1 did not display significant ACAT activity. None of the enzymes showed the ability to use ergosterol, the major sterol in yeast microsomes, as a substrate (data not shown). While the ACAT1 and ACAT2 mediated activities were equally sensitive (75% inhibition) to the ACAT inhibitor Dup128 (0.5 μM; not shown), they showed significantly different sensitivity to the histidine/tyrosine modifying agent diethylpyrocarbonate (DEPC, Table I). This reagent was previously demonstrated to distinguish liver and adrenal ACAT activities, the latter being significantly more sensitive. Since adrenal ACAT would primarily represent ACAT1, our data are consistent with ACAT2 representing the DEPC-resistant isoform identified by Kinnunen *et al.* (2).

DISCUSSION

We have isolated two independent human cDNAs, ARGP1 and ACAT2, which encode proteins with significant sequence similarities to human ACAT1. The level of nucleotide sequence conservation between ACAT1 and ACAT2 (55%) suggests their common evolution possibly arising from a gene duplication event, as clearly occurred in the case of the yeast *ARE* gene family. However, ARGP1 is more distantly related, bearing 39 and 43% nucleotide identity with ACAT1 and ACAT2, respectively, and may have evolved independently. The uniform similarity between the human genes and the two yeast *ARE* genes precludes any assignment of lineage across species.

The similarity among the three human ACAT-like proteins is most distinct over their COOH-terminal regions just as is the case when comparing the yeast Are proteins to ACAT1. The predicted ARGP1 protein displays 28% identity with ACAT1 over this portion of the molecule and includes a FY.DWWN motif present in all cloned ACATs and shown to be important for enzymatic activity (Fig. 7A).[3] However, ARGP1 is the most divergent member of this gene family. For example, a HSF motif (residues 268–270) is invariant in ACAT1 and yeast Are enzymes and was critical to ACAT1 activity in CHO cells. Replacement of Ser by Leu produced an inactive and unstable molecule (33). This motif is not conserved in ARGP1, although several serines are present in the region (*e.g.* Ser^{227}, Fig. 7*B*). ARGP1 is also unique in its predicted possession of a diacylglycerol/phorbol ester-binding site (Fig. 7*A*), leading us to speculate that this enzyme might esterify diacylglycerol to produce triglyceride. Sequence similarity between diacylglycerol acyltransferase and ACAT enzymes might be expected since both have a common substrate, acyl-CoA, but differ in the alcohol (cholesterol or diacylglycerol) used as a second substrate.

Of the two new gene products described here, ACAT2 displays significantly greater sequence similarity to ACAT1, with an overall identity of 47% and 63% invariance over the COOH-terminal half of the molecules. The FY.DWWN motif common to this family of proteins is maintained in ACAT2 to the extent that the flanking residues render the tyrosine a candidate for phosphorylation as observed in ACAT1 and in yeast (Fig. 7*A*). Tyrosine phosphorylation may be a regulator of ACAT activity, although serine and threonine phosphorylation is unlikely to be involved (34, 35). The HSF motif found in ACAT1, Are1 and Are2 is conservatively replaced in ACAT2 by YSF (residues 244–246; Fig. 7*B*). Interestingly, histidine modifying agents selectively inactivate adrenal microsomal ACAT activity but display a significantly higher K_i (1500 *versus* 250 μM) against liver microsomes (2). It is intriguing to speculate that sequence variation in the (H/Y)SF motif may explain this observation. In accordance with this, we showed that ACAT1 was significantly more sensitive to DEPC than ACAT2. In common with ACAT1, Are1 and Are2, the ACAT2 sequence predicts a leucine heptad motif which may play a role in multipeptide complex formation. Radiation inactivation studies in rat liver microsomes have shown that the ACAT enzymatic complex is about 200 kDa (36, 37), much larger than the predicted monomer for ACAT 1 (65 kDa) or ACAT2 (60 kDa). There is also evidence that ACAT1 interacts with itself in a yeast two-hybrid system (38) and ACAT2 may be similar in this regard. ARGP1 and ACAT2 are also similar to ACAT1 in terms of hydrophobicity. While previous studies suggested that ACAT1 contains two transmembrane domains (6), the PredictProtein algorithm (39) indicates

[3] Z. Guo, D. Cromley, J. T. Billheimer, and S. L. Sturley, manuscript in preparation.

A The "DWWN" region of the ACAT gene family

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ARE1  482   ELtRFaDRyF YgDWWNcvSf eeFsRiWNVP VHKfLlRHVY hssmgal.hl sKS
ARE2  514   ELtRFGDRyF YgDWWNcvSw adFsRiWNiP VHKfLlRHVY hssmssf.kl nKS
ACAT1 394   EmlRFGDRmF YkDWWNStSy snyyRtWNVv VHdWLyyyaY kdfLwffskr fKS
ACAT2 368   EmlRFGDRmF YrDWWNStSf snyyRtWNVv VHdWLysyVY qdgLrllgar arg
ARGP1 351   ELmqFGDReF YrDWWNSeSv tyFwqnWNiP VHKWciRHfY kpmLrrgss. .Kw
                      π                              *   *
Consensus   EL-RFGDR-F Y-DWWNS-S- --F-R-WNVP VHKWL-RHVY ---L------ -KS
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B The "HSF" region of the ACAT gene family

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ARE1  306   FvMKSHSFAf yNgyLWdIkq
ARE2  335   lLMKmHSFAf yNgyLWgIke
ACAT1 265   FvMKaHSFvr eNvprvLnsA
ACAT2 240   FLMKSySFlr eavpgtL.rA
ARGP1 206   FL.KlfSyrd vNswcr..rA
                    σ
Consensus   FLMKSHSFA- -N--LWLI-A
```

FIG. 7. **Consensus sequences in the ACAT multigene family.** Two regions of structural and functional conservation are shown. The amino acid position of each initial residue is shown. *Uppercase* residues indicate those of the consensus calculated with a plurality of 2. *A*, The DWWN region. The FY.DWWN motif is invariant in all members identified to date of this gene family, the tyrosine and tryptophans being critical to activity.[3] In all but ARGP1, the Tyr constitutes a candidate target for phosphorylation (indicated in *bold* and by π). In ARGP1, the *underlined* sequence HKWCIRHFYKP represents a candidate for diacylglycerol binding as found in protein kinase C and diacylglycerol kinases (motif, H.[FWY]..[KR].F..P). The *asterisks* identify those residues critical to definition of this motif that distinguish ARGP1 from the other members of the family. *B*, the HSF region. The central serine residue (indicated σ) was found to be critical to the activity and stability of Chinese hamster ovary ACAT1.

eight such domains in ACAT1, similar to the number predicted for ARGP1 (nine) and ACAT2 (seven). Membrane spanning domains are expected characteristics of ACAT and diacylglycerol acyltransferase enzymes since both activities are associated with microsomal membranes (40–42).

In addition to sequence similarity with ACAT1, we expect alternate ACAT enzymes to be expressed in the tissues which retain ACAT activity in the induced mutant ACAT1 mouse, namely the liver and intestine. ARGP1 met this criteria, however, it is also highly expressed in human adult adrenal cortex which was depleted of cholesterol esters in the induced mutant mouse. Monocytes from *acact*$^-$ mice were also devoid of cholesterol ester and yet ARGP1 mRNA was detected in the human THP1 monocyte cell line. This evidence is contrary to ARGP1 being an ACAT, barring species-specific differences in expression. By the sensitive technique of RT-PCR, ACAT2 expression was observed in human adult liver and intestine and in cell culture models of the hepatocyte and intestinal enterocyte but was undetectable in THP1 monocytes and macrophages. This profile of expression is consistent with a role for ACAT2 in the livers and intestine of mammals, particularly ACAT1 knockout mice.

In confirmation of ACAT2 being a candidate for a second ACAT, heterologous expression of ACAT2 in an ACAT-negative yeast strain conferred significant microsomal cholesterol esterification with oleoyl-CoA at a level comparable to the 20–50 pmol/min/mg of protein observed in human liver microsomes supplied with exogenous cholesterol (43). The ACAT2-mediated esterification activity was significantly (85%) less than that mediated by ACAT1 in yeast. This may be due to differences in protein expression (although both mRNAs were produced at high levels as detected by RT-PCR, data not shown), protein stability, or a genuine difference between the two enzymes.

Liver ACAT, predicted to comprise both ACAT1 and ACAT2, utilizes a limited range of sterol substrates but a wide variety (16:0, 18:0, 18:1, 18:2, and 20:4) of fatty acyl-CoAs (27, 44). Determining substrate-specific differences between ACAT1 and ACAT2 may thus explain their redundancy. The redundancy may also be related to substrate affinity such as seen between the hexokinase types I-III and hexokinase type IV (glucokinase) (45). In such a scenario, one ACAT would have a lower affinity for cholesterol and only catalyze esterification at high cholesterol concentrations.

In addition to potential differences in activity, the two enzymes may have different physiological roles. For storage, cholesterol esters concentrate as cytoplasmic neutral lipid droplets, whereas for lipoprotein synthesis, cholesterol esters are incorporated into lipoprotein particles in the endoplasmic reticulum lumen. Redundant ACAT enzymes might allow one to be specific for cytoplasmic release of the cholesterol ester product and another to mediate endoplasmic reticulum lumenal release. Since lipoprotein synthesis occurs primarily in the liver and intestine, we speculate that ACAT2 may release cholesterol ester into the endoplasmic reticulum lumen, leaving ACAT1 to esterify and store sterols in the cytoplasm. The large amount of cholesterol ester, likely as cytoplasmic droplets, in the livers of high fat, high cholesterol fed *acact*$^-$ mice, is contrary to this hypothesis. Alternatively, ACAT2's role may be important in the fetus since it was easily detected by Northern blot in human fetal liver.

The abundance of ARGP1 entries in the dbEST from a wide variety of cDNA libraries is reflective of the ubiquitous nature of ARGP1 expression in human adult tissues and tissue culture cell lines. This suggests that ARGP1 serves a function important to many cell types. Expression of two independent clones of ARGP1 under the regulation of two yeast promoters, *GAL1/10* and *GAPDH* (not shown), failed to detectably esterify cholesterol or ergosterol. ARGP1-specific mRNA was identified by RT-PCR in each case. We take this as further evidence that unlike ACAT1 and ACAT2, ARGP1 is not involved in cholesterol esterification, at least when expressed in yeast. Based on the conservation of amino acids in ARGP1 that are important for ACAT1 to be active, ARGP1 likely catalyzes a reaction similar to ACAT. Other esterification reactions which use fatty-acyl CoAs as substrates include retinol esterification, methyl ester formation, triterpene esterification, monoacylglycerol transferase, and diacylglycerol transferase. In the latter case our observations of a diacylglycerol-binding site in ARGP1 biases us to the possibility of ARGP1 being diacylglycerol acyltransferase, which to date has not been isolated at the molec-

ular level. We are presently investigating whether ARGP1 can mediate these reactions.

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Alteration of Seed Fatty Acid Composition by an Ethyl Methanesulfonate-Induced Mutation in *Arabidopsis thaliana* Affecting Diacylglycerol Acyltransferase Activity[1]

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In characterizing the enzymes involved in the formation of very long-chain fatty acids (VLCFAs) in the Brassicaceae, we have generated a series of mutants of *Arabidopsis thaliana* that have reduced VLCFA content. Here we report the characterization of a seed lipid mutant, AS11, which, in comparison to wild-type (WT), has reduced levels of 20:1 and 18:1 and accumulates 18:3 as the major fatty acid in triacylglycerols. Proportions of 18:2 remain similar to WT. Genetic analyses indicate that the fatty acid phenotype is caused by a semidominant mutation in a single nuclear gene, designated *TAG1*, located on chromosome 2. Biochemical analyses have shown that the AS11 phenotype is not due to a deficiency in the capacity to elongate 18:1 or to an increase in the relative Δ15 or Δ12 desaturase activities. Indeed, the ratio of desaturase/elongase activities measured in vitro is virtually identical in developing WT and AS11 seed homogenates. Rather, the fatty acid phenotype of AS11 is the result of reduced diacylglycerol acyltransferase activity throughout development, such that triacylglycerol biosynthesis is reduced. This leads to a reduction in 20:1 biosynthesis during seed development, leaving more 18:1 available for desaturation. Thus, we have demonstrated that changes to triacylglycerol biosynthesis can result in dramatic changes in fatty acid composition and, in particular, in the accumulation of VLCFAs in seed storage lipids.

The fatty acyl composition of seed TAGs determines their physical and chemical properties and, thus, their use in edible oil or industrial applications. TAG composition depends on the interaction of several different groups of enzymes in the lipid biosynthesis pathway. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids up to and including oleic acid. Modifying enzymes, such as the extraplastidic Δ12 and Δ15 desaturases, elongases, hydroxylases, and epoxidases, yield polyunsaturated, very long-chain, hydroxy-, and epoxy- fatty acids, respectively. Acyltransferases insert specific acyl moieties onto the glycerol backbone to yield TAGs via the Kennedy pathway (Murphy, 1993; Ohlrogge, 1994).

Most oilseed crops accumulate a limited range of fatty acids in their seed oil. Just six fatty acids contribute more than 95% of world production (Schmid, 1987). Many other fatty acids are of considerable interest as renewable feedstocks for chemical industries. These include the VLCFAs, behenic (20:0), eicosenoic (20:1), and erucic (22:1), characteristic of the seed oils of a number of species within the Brassicaceae. There are currently more than 1000 patented applications for C_{22} oleochemicals and their derivatives (Sonntag, 1991; Taylor et al., 1992c; N.O.V. Sonntag, personal communication). Research to maximize the VLCFA content of industrial rapeseed, for example, is an important effort being pursued in several biotechnology and breeding laboratories (Taylor et al., 1992c; Murphy, 1993).

In the course of characterizing the enzymes involved in the formation of VLCFAs in the Brassicaceae, we have generated a series of mutants of *Arabidopsis thaliana* that are deficient in the accumulation of VLCFAs (Kunst et al., 1989, 1992a, 1992b). Detailed analyses of these mutants contribute to our understanding of the organization of the elongases and their role in the lipid bioassembly pathway. Furthermore, with the development of *Arabidopsis* as a model organism for plant molecular genetics, it is possible to clone genes in the lipid pathway affected by mutations, using the techniques of chromosome walking from RFLP

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Abbreviations: ACCase, acetyl-CoA carboxylase (EC 6.4.1.2); ACP, acyl carrier protein; cM, centiMorgan; DAG, *sn*-1,2-diacylglycerol; DGAT, *sn*-1,2 diacylglycerol acyltransferase (EC 2.3.1.20); Δ15 desaturase, linoleate (ω-3) desaturase; Δ12 desaturase, oleate (ω-6) desaturase; d.p.a., days postanthesis; EMS, ethyl methanesulfonate; FAME, fatty acid methyl ester; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); KAS II, 3-ketoacyl-ACP synthetase II; LPA, lyso-phosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; RFLP, restriction fragment length polymorphism; TAG, triacylglycerol; VLCFA, very long-chain ($>C_{18}$) fatty acid; WT, wild type; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid, *cis*-Δ9-octadecenoic acid; 18:2, linoleic acid, *cis*-Δ9, Δ12-octadecadienoic acid; 18:3, α-linolenic acid, *cis*-Δ9, Δ12, Δ15-octadecatrienoic acid; 20:0, eicosanoic acid; 20:1, *cis*-Δ11-eicosenoic acid; 20:2, *cis*-Δ11, Δ14 eicosadienoic acid; 22:0, docosanoic acid; 22:1, erucic acid, *cis*-Δ13-docosenoic acid.

sites (Meyerowitz, 1987; Arondel et al., 1992; Dean, 1993) or T-DNA tagging (Dean, 1993; Yadav et al., 1993). One mutant, AC56, has been shown to be deficient in elongase activity (Kunst et al., 1989, 1992b). Here we report the detailed characterization of another mutant, AS11, which has reduced 20:1 and 18:1 and, instead, accumulates 18:3. The results suggest that, rather than a deficiency in the capacity to elongate 18:1, this mutant has a reduced diacylglycerol acyltransferase activity that results in modulation of 20:1 biosynthesis. Thus, we have shown for the first time in *A. thaliana* that changes to lipid bioassembly at the level of the Kennedy pathway can result in dramatic changes in extraplastidic fatty acid modification.

MATERIALS AND METHODS

Plant Material

Several populations of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia; WT) were mutagenized separately by soaking the seeds (M_1) for 16 h in 0.3% (v/v) EMS as described by Haughn and Somerville (1986). M_1 seeds were germinated, and plants were grown to maturity and allowed to self-fertilize to produce M_2 seeds. Approximately 9000 randomly chosen M_2 seeds were planted, and plants were again self-fertilized and then harvested individually to give several thousand M_3 families. M_3 seed samples (20–30 seeds) were treated with methanolic HCl (see below) to digest the tissue and convert the fatty acids present in the seed oil to the corresponding FAMEs. The FAME samples were then screened by GC for changes in acyl composition as described by Kunst et al. (1992b). Families with altered VLCFA content were retained. Fifteen seeds from each potentially interesting M_3 family were planted and harvested separately. The FAME composition of a number of M_4 seeds from each of the 15 M_3 plants was analyzed to determine whether the trait was stably inherited and was segregating and to isolate a homozygous line. Selected lines were backcrossed to the WT at least twice before being used for analyses. All plants were grown in growth chambers under continuous fluorescent illumination (150–200 $\mu E\ m^{-2}\ s^{-1}$) at 22°C on Terra-lite Redi-earth (W.R. Grace and Co., Canada Ltd., Ajax, ON, Canada). Under these growth conditions, one generation took 8 to 10 weeks.

Linkage tester lines in ecotype Landsberg *erecta* were utilized in preliminary investigations to map the mutation. The W-100 line contained two visible markers for each of the five *A. thaliana* chromosomes (Koornneef and Hanhart, 1983; Koornneef et al., 1983, 1987). Line PBI1 contained multiple chromosome 2 markers *sti*, *cp2*, *er*, *as*, and *cer8*. The genetic distances between the various loci were assessed with the computer program LINKAGE-1 (Suiter et al., 1983). The estimates of the recombination percentages (r) for double crossovers were corrected using the Kosambi mapping function, converting them to map distances (D) in cM: $D = 25\ln(100 + 2r/100 - 2r)$ with $SD = 2500s_r/2500 - r^2$ (where s_r is the SD of r) (Koornneef et al., 1983).

Studies of the germination responses of WT and AS11 seeds to exogenously supplied ABA were performed as described by Finkelstein and Somerville (1990), and tests of seed dormancy using developing seeds isolated from WT and AS11 siliques were conducted as described by Karssen et al. (1983).

Substrates and Reagents

[1-^{14}C]oleic acid (58 mCi $mmol^{-1}$) was purchased from Amersham Canada, Ltd. (Oakville, ON), and [1-^{14}C]erucic acid (52 mCi $mmol^{-1}$), [1-^{14}C]sodium acetate (59 mCi $mmol^{-1}$), and L-α-palmitoyl-2-[1-^{14}C]oleoyl-PC (58 mCi $mmol^{-1}$) were purchased from NEN Research Products (Mississauga, ON). [1-^{14}C]Eicosenoic acid was synthesized as described previously (Kunst et al., 1992b). 1-^{14}C-labeled fatty acids were converted to the corresponding acyl-CoA thioesters using the method described by Taylor et al. (1990). [1-^{14}C]16:0-ACP (56 mCi $mmol^{-1}$) was synthesized and kindly provided by Dr. John Ohlrogge (Michigan State University). Specific activities were adjusted as required by diluting with authentic unlabeled standards. Unlabeled acyl-CoAs, sodium acetate, ATP, CoA-SH, NADH, NADPH, polyvinylpolypyrrolidone, (±)-ABA, polar lipid standards, and most other biochemicals were purchased from Sigma. Neutral lipid standards were obtained from NuChek Prep, Inc. (Elysian, MN), and FAME standards were supplied by Supelco Canada, Ltd. (Oakville, ON). Mixed TAG and DAG standards for GC, which were not commercially available, were synthesized from the corresponding DAGs or MAGs by condensation with the appropriate acyl chloride and purified as described by Taylor et al. (1991). HPLC-grade solvents (Omni-Solv, BDH Chemicals, Toronto, ON) were used throughout these studies.

Harvests of Developing and Mature Seed, Leaf, and Root Tissue

In all analyses discussed hereafter, the stages of seed development examined (in d.p.a) are described as: "milky" (11 d.p.a. for both WT and AS11), "early green" (13 d.p.a. for WT, 16 d.p.a. for AS11), "green" or "mid-development" (15 d.p.a. for WT, 21 d.p.a. for AS11), "green-brown" (19 d.p.a. for WT, 25 d.p.a. for AS11), and "mature" dry (21 d.p.a. for WT, 28 d.p.a. for AS11). Developing seeds at the desired stages were harvested from siliques and used immediately or frozen in liquid nitrogen for further analysis. Root tissue was obtained from 4-week-old plants grown in liquid culture using a protocol adapted from Valvekens et al. (1988). Fresh leaf tissue was harvested from 4-week-old seedlings and FAME analyses were performed immediately.

Fatty Acid and Lipid Analyses

In some cases, lipids were saponified by treatment of tissues or reaction mixtures directly with 10% methanolic-KOH at 80°C for 2 h. The samples were then acidified with 6 N HCl, and the free fatty acids were extracted with hexane. Following removal of the hexane, the fatty acids were converted to their methyl esters for GC analysis. For FAME analyses, *A. thaliana* M_4 seed, leaf, or root tissues, intact lipid species, or saponified free fatty acid samples were treated with 3 N methanolic-HCl (Supelco Canada,

Ltd.), at 80°C for 2 h after the addition of 1 to 10 μg of 17:0 free fatty acid as an internal standard. FAMEs were extracted and analyzed by GC on a DB-23 column as described previously (Kunst et al., 1992b).

Total lipid extracts were prepared from seeds at the milky and green stages of development and from mature seed, and individual polar and neutral lipid classes were separated and recovered as described by Taylor et al. (1991, 1992a). Following purification by chromatography on silica gel G columns, TAG or DAG samples were spiked with trierucin or 1,2-dierucin as the internal standard, respectively. TAG and DAG separations were performed using a 3-m-wide bore DB-1 column on a Hewlett-Packard 5890 series II GC under the following conditions: injector temperature, 350°C; detector temperature, 375°C; program: 200 to 350°C at 10°C/min, then isothermal for 5 to 10 min; column flow 10 mL/min at a split vent ratio of 10:1. Acyl composition assignments were based on retention times of standards and confirmed by MS. Intact TAGs were analyzed by NH_4^+-Cl-MS and product ion MS/MS analyses as described by Taylor et al. (1995). Stereospecific analyses of TAGs were performed using both pancreatic lipase and a Grignard-based method in which MAGs were generated, followed by a chiral separation of di-dinitrophenylurethane-MAG derivatives as reported previously (Taylor et al., 1994, 1995).

Acetate Labeling (in Vivo Studies)

Developing seeds at the green mid-development stage were harvested from 50 siliques and placed immediately in beakers chilled on ice. Intact seeds of a known fresh weight were placed in 10-× 16-mm glass tubes and 40 μM [1-^{14}C]sodium acetate (59 nCi nmol^{-1}), 100 mM Hepes-NaOH, and 320 mM Suc were added to a final volume of 2 mL at pH 7.4. Tubes were shaken at 100 r.p.m. and 30°C under incandescent light (10,000 Lux at the level of the seeds) for 18 h. Following incubation, the medium was aspirated away, seeds were washed several times with 2-mL aliquots of distilled water, and then lipids were saponified or extracted and analyzed as described above.

In Vitro Studies

Cell-free homogenates were prepared from developing green WT and AS11 seeds as described by Kunst et al. (1992b) and adjusted to equivalent protein concentrations with grinding medium. Elongase assays were performed using 40 μM [1-^{14}C]oleoyl-CoA (10 nCi nmol^{-1}) and 1 mM malonyl-CoA with all other reaction conditions as described by Kunst et al. (1992b). Desaturase assays were performed by incubating 150 to 250 μg of homogenate protein with 75 mM Hepes-NaOH, pH 7.4, 1 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM CoA-SH, 1 mM NADH, and 40 μM [1-^{14}C]oleoyl-CoA (10 nCi nmol^{-1}) or 10 μM L-α-palmitoyl-2-[1-^{14}C]oleoyl-PC (58 nCi nmol^{-1}), in a final volume of 0.5 mL in open glass tubes for 30 min at 30°C and 100 r.p.m. For the comparative in vitro elongase and desaturase assays, homogenates prepared from developing green seeds of lines AC56 (elongase mutant) and AJ70 (Δ15 desaturation mutant) were used as controls. KAS II assays were conducted by monitoring the conversion of [1-^{14}C]16:0-ACP (56 nCi nmol^{-1}) to [1-^{14}C]18:0-ACP in the presence of 100 μM malonyl-CoA, 0.5 mM NADH, 0.5 mM NADPH at 30°C and 100 r.p.m., adapted from the method reported by Jaworski et al. (1974). Radiolabeled fatty acyl products of the elongase, desaturase, and β-KAS-II reactions were isolated by saponifying the reaction mixture and converting the fatty acids to FAMEs for analysis by radio-HPLC as described by Kunst et al. (1992b). ACCase assays of developing seeds were carried out using the extraction and assay methods described by Parker et al. (1990). Biosynthesis of glycerolipids via the Kennedy pathway was monitored by supplying homogenates prepared from green developing seeds with G-3-P and radiolabeled acyl-CoAs, followed by separation and quantitation of lipid species as described previously (Taylor et al., 1991, 1992a). Direct assays of DGAT activity were performed by supplying homogenates prepared from developing seeds at various developmental stages with emulsified 1,2-diolein as acyl acceptor, and various [1-^{14}C]acyl-CoAs as acyl donors, followed by radio-HPLC measurement of intact labeled TAGs as described by Weselake et al. (1991). Homogenate protein concentrations were measured by the method of Bradford (1976).

Northern Analyses for Δ12 Desaturase and Δ15 Desaturase Gene Expression

Total RNA was extracted from green developing WT and AS11 seeds using the method of Lindstrom and Vodkin (1991). RNA samples were denatured with formaldehyde and separated on 1.2% formaldehyde agarose gels. The amount of total RNA loaded per lane was calibrated by the ethidium bromide-staining intensity of the rRNA bands. The RNA was transferred onto a Zeta Probe nylon membrane (Bio-Rad) and hybridized with ^{32}P-labeled Δ12 or Δ15 desaturase probes according to the manufacturer's protocol.

A plasmid pBNDES3 carrying a cDNA clone of the *Brassica napus* homolog of the *Arabidopsis fad3* (Δ15 desaturase) gene was obtained from the Arabidopsis Biological Resource Center at the Ohio State Biotechnology Center (Columbus, OH). The open reading frame of the *fad2* (Δ12 desaturase) gene (Okuley et al., 1994) was PCR amplified from *A. thaliana* DNA (using the oligonucleotides GCCGAATTCATGGGTGCAGGTGGAAGA and GCCGAATTCCACCATCATGCTCATAACT) and cloned into the plasmid pSE936 (Elledge et al., 1991). The cDNA inserts were excised, and the DNA fragments were purified with the GENECLEAN II kit (Bio 101 Inc., LaJolla, CA), labeled with ^{32}P using the Gibco BRL Random Primers DNA Labeling System as described by the manufacturer (Gibco BRL Life Technologies), and used as probes in northern analyses.

RESULTS AND DISCUSSION

Mutant Isolation and Fatty Acid and Genetic Analyses

Direct GC analysis of 2000 M_3 families resulted in the isolation of 35 mutants with altered seed fatty acid com-

position. Most of the mutants exhibited one of three distinct phenotypes: deficiencies in VLCFA biosynthesis (line AC56), Δ12 desaturation (line AL63), or Δ15 desaturation (line AJ70). The biochemical and genetic characterization of mutant AC56 and its allelles (mutants AK57, AT59, and AX33) was reported previously (Kunst et al., 1992a, 1992b; Taylor et al., 1992b). In AC56, a single semidominant nuclear mutation in the *FAE1* gene resulted in a deficiency in acyl chain elongation from 18:1 to 20:1 and 22:1 and from 18:0 to 20:0. As a result, the proportion of VLCFAs decreased and that of 18:1 increased dramatically.

In the present study, an unusual mutant, AS11, originally reported in 1989 (Kunst et al., 1989), was further characterized. AS11 had much reduced proportions of 20:1 and 18:1, whereas the proportion and absolute amounts of 18:3 dramatically increased (Fig. 1). The proportion of 18:2 remained essentially identical with that of WT. In contrast to the AC56 mutant, the levels in AS11 of the other VLCFAs, 20:0, 22:0, and 22:1, were not reduced, and 20:2 was, in fact, significantly elevated. The ratio of 18:3/18:2 in AS11 seed was 1.39 ± 0.02, whereas in WT seed, it was 0.65 ± 0.01. In having an elevated 18:3/18:2 ratio, AS11 is somewhat similar to a mutant JB11 (*ela1*, enhanced linolenate accumulation; 18:3/18:2 ratio 1.04 ± 0.10) previously described by Lemieux et al. (1990). However, in contrast to JB11, AS11 has a drastically reduced proportion of 20:1; in JB11 this fatty acid is unaffected. Furthermore, in the present study, the mutation in AS11 was found to be seed specific, with no significant effect on the fatty acid composition of leaf or root lipids (data not shown). In WT and AS11 leaves, the 18:3/18:2 ratios were 3.30 ± 0.21 and 3.46 ± 0.11, respectively, whereas in WT and AS11 roots, the respective 18:3/18:2 ratios were 0.95 ± 0.05 and 0.99 ± 0.02. In contrast, JB11, in addition to altered 18:3/18:2 seed ratios, showed small but statistically significant increases in the 18:3/18:2 ratio in leaf and root tissues, relative to WT (Lemieux et al., 1990).

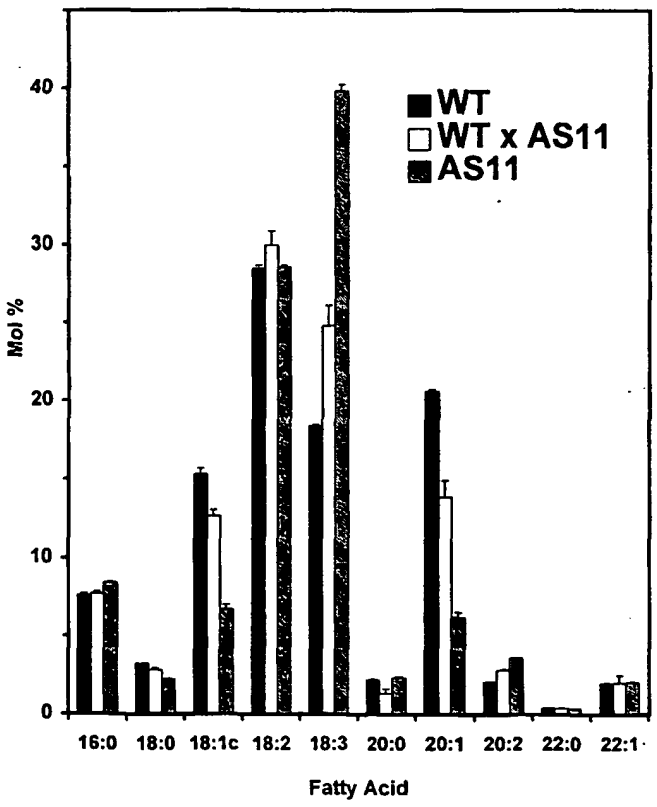

Figure 1. Fatty acid composition of total lipids in mature *A. thaliana* seed of WT, AS11, and WT × AS11 F_1 progeny. Values are shown ± SE (n = 12). 18:1c is primarily the *cis*-Δ9 isomer, oleic acid (greater than 90%) plus a small amount (less than 10%) of the *cis*-Δ11 isomer, vaccenic acid.

To determine the inheritance of the altered fatty acid composition, 10 M_3 seeds of AS11 were sown, plants were grown to maturity and allowed to self-fertilize, and M_4 seeds were harvested and analyzed by GC. The fatty acyl composition of the AS11 M_4 seeds was virtually indistinguishable from that of the M_3 seeds, indicating that the altered fatty acid composition was heritable and that the original AS11 M_3 line was homozygous.

To determine the genetic basis for the alteration in fatty acid composition in AS11, reciprocal crosses were made to WT, and the F_1 seeds were analyzed. The F_1 seeds had intermediate levels of 20:1, 18:1, 18:3, and most other fatty acids, relative to that of the parental lines (Fig. 1, WT × AS11). The intermediacy of these values indicates partial dominance and suggests a gene dosage effect, i.e. that the amount of available gene product is limiting in AS11, resulting in these phenotypic changes in fatty acid proportions. The F_2 progenies derived from self-fertilization of several F_1 plants followed a 1:2:1 segregation pattern (WT × AS11, 56:132:70; χ^2 = 1.42; $P > 0.5$; degrees of freedom = 2), as expected if the altered fatty acid composition is caused by a single nuclear mutation in a gene we designate *TAG1*.

Complementation tests between AC56 and AS11 clearly indicated that the AS11 mutant represents a different complementation group, suggesting that the elongation of 18:1 in seeds is controlled by several loci. Indeed, two other mutants with reduced capacity to elongate 18:1, AK9 and BB6, were previously shown to be nonallellic, their fatty acid phenotypes being due to mutations in two separate genes that were also distinct from the gene interrupted by the mutation in line AC56 (Kunst et al., 1992b). In preliminary experiments to determine the chromosomal location of the lesion in AS11, the mutant was crossed to a multiply marked line (W-100) containing two visible markers for each of the five *A. thaliana* chromosomes (Koornneef et al., 1983, 1987). An analysis of the F_2 progeny indicated that the lesion was located on chromosome 2. This result was confirmed by analyzing F_2 progeny of a cross between the AS11 mutant and PBI1, a line that carries multiple chromosome 2 phenotypic markers: *sti*, *cp2*, *as*, *er*, and *cer-8*. Examination of 317 F_2 plants indicated that the mutation in AS11 lies in the region between the *sti* and *cp2* morphological markers, approximately 17.5 ± 3 cM from the *sti* locus and 8 ± 2 cM from the *cp2* locus. Thus, the AS11 locus is distinctly different from *FAE1*, which was shown to be about 11 cM from the *cer2* locus on chromosome 4 (Kunst et al., 1992a). However, since there is no indication that loci

belonging to specific phenotypic groups are preferentially located on specific chromosomes (Koornneef et al., 1983), it is not unexpected that loci affecting VLCFA content may be found on both chromosomes 2 and 4.

Biochemical and Analytical Characterization of the AS11 Mutant

It was observed that, although plant growth, flowering, and self-fertilization were normal in AS11, seed development was significantly retarded. Whereas WT seed was mature at 21 d.p.a., the AS11 mutant siliques were green and the seeds were still developing, maturing a full 1 week later (28 d.p.a.). In *Arabidopsis* as in other oilseeds, the major accumulation of lipid is known to occur in a well-defined and relatively short period during middle to late embryogenesis (Mansfield and Briarty, 1992). This period of maximal lipid deposition (15–18 d.p.a in WT) was also delayed in AS11 (21–25 d.p.a.). In addition, the total lipid content in mature AS11 seed (195 ± 9 μg/mg dry weight) was significantly reduced, reaching only about 75% of that observed in mature WT seed (262 ± 8 μg/mg dry weight). The fatty acid phenotype of AS11 mature seed lipids showed marked changes in the proportions of several fatty acids: 20:1 and 18:1 decreased by about 60%, whereas the proportions of 18:3 increased by 100% (Fig. 1). Similar trends were observed in the absolute amounts of these fatty acids (μg/mg dry weight) accumulating in mature seed lipids (Fig. 5C). Mature seed weights (2.00 ± 0.05 mg/100 seeds for both WT and AS11) and protein levels (WT, 106 ± 4 μg/mg dry weight; AS11, 112 ± 6 μg/mg dry weight) were not significantly different. Fresh weights and total protein for developing seed at each stage of development were comparable in WT and AS11, varying by ≤10%.

To examine fatty acid biosynthesis in vivo, fatty acid analyses were performed on total lipid fractions isolated from intact green seeds, harvested from siliques of the WT and AS11 lines at mid-development (Fig. 2A). AS11 showed a reduced proportion of 20:1 and 18:1 and an increased proportion of 18:3. Seeds at this stage of development were also incubated with [^{14}C]acetate and the ^{14}C-labeled de novo synthesized fatty acids were assayed (Fig. 2B). Relative to WT, in AS11 the proportion of [^{14}C]20:1 was decreased, whereas the proportion of [^{14}C]18:2 and 18:3 were increased slightly, although not yet at significant expense to the proportion of [^{14}C]18:1. The latter observation is consistent with the known sequential desaturation of 18:1 first to 18:2 and then 18:3. However, it does indicate that a strong decrease in the level of newly synthesized 20:1 was observed prior to a marked increase in the level of 18:3. Of equal importance, the overall level of [^{14}C]acetate incorporated into fatty acids (dpm/mg fresh weight of developing seed) was lower in AS11, averaging only 66% of the rate of incorporation observed in WT embryos. This finding is consistent with the lower lipid content in mature AS11 seed.

In having altered seed development and reduced levels of 20:1, AS11 is somewhat similar to the ABA-insensitive *A. thaliana* mutant *ABI3* (Finkelstein and Somerville, 1990). However, AS11 is also characterized by an accumulation of

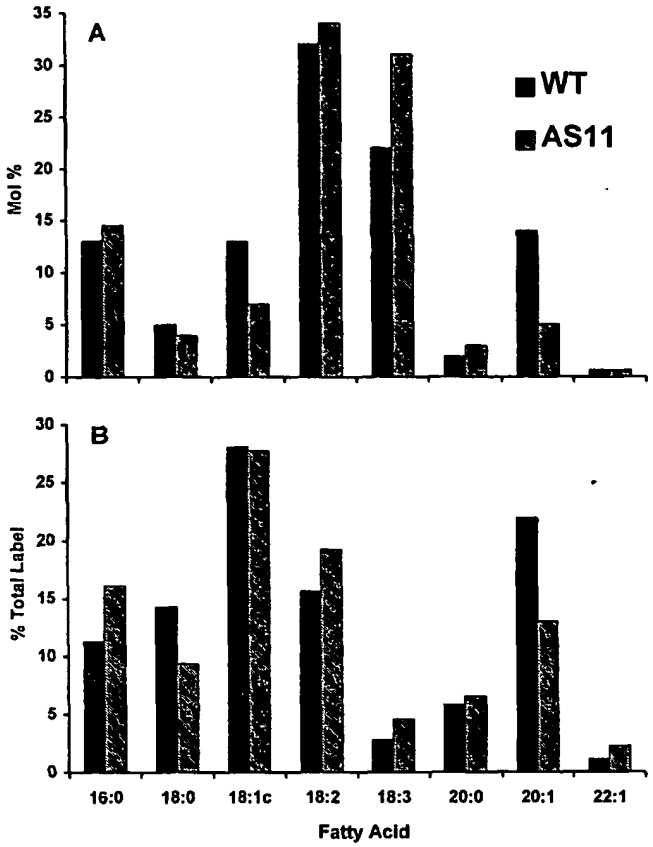

Figure 2. A, Fatty acid composition (mol %) of total lipid extracts from WT and AS11 green seeds at mid-development. B, Proportions of ^{14}C-labeled fatty acids (% total label) produced during an 18-h incubation of WT and AS11 green seeds at mid-development with [1-^{14}C]sodium acetate as described in "Materials and Methods."

18:3 as the major fatty acid, reduced levels of 18:1, and reduced storage lipid in mature seed, whereas in *ABI3*, 18:2 was the major fatty acid, 18:1 was increased, and there was no significant effect on total fatty acid content. In addition, a major characteristic of all ABA-insensitive (*ABI*) mutants is the ability of mature seeds to germinate in the presence of concentrations of exogenous ABA that fully inhibit germination in WT seeds (Finkelstein and Somerville, 1990). However, the inhibition of AS11 seed germination by ABA was similar to that of WT seeds, both being fully inhibited by 3 μM exogenous ABA. Neither did AS11 display any of the phenotypic characteristics of ABA-deficient mutants (nondormant seeds; precocious germination in siliques; Koornneef et al., 1982; Karssen et al., 1983). Dormancy tests in milky, green, and green-brown developing seeds isolated from siliques of AS11 and incubated under conditions of high RH, were negative. The AS11 locus on chromosome 2 is different from the known loci for *A. thaliana* ABA-insensitive mutants (*ABI1*, *ABI2*, and *ABI3*, on chromosomes 4, 5, and 3, respectively) and ABA-deficient mutants (chromosome 5) (Koornneef et al., 1982, 1984). Collectively, these data suggest that the AS11 mutation is not related to ABA-deficient or ABA-insensitive *A. thaliana* mutants.

Based on the fatty acid profile of mature (Fig. 1) and developing (Fig. 2) seed of AS11, a lesion in one of at least

five different biochemical steps of fatty acid synthesis/modification was suspected. Since 20:1 was reduced but 18:3 was elevated, the primary candidates were altered 18:1-CoA elongase or Δ15 or Δ12 desaturase activities. Changes in the activity of KAS II, which converts palmitoyl-ACP to stearoyl-ACP in seed plastids, may have caused the minor changes in the ratio of 16:0/18:0. A lesion in extraplastidial (cytosolic) ACCase (Page et al., 1994; Roesler et al., 1994) was a remote possibility but unlikely, since the proportions of other VLCFAs, which also require malonyl-CoA for chain extension, were not adversely affected in AS11.

The results of in vitro biochemical assays performed with homogenates from green developing WT and AS11 seeds at mid-development are summarized in Table I. Similar trends were also observed when data were expressed on a seed fresh weight basis (data not shown). Rather unexpectedly, the elongase activity in AS11 was about 2-fold higher than the corresponding activity in WT seeds. As suspected, the ACCase activity in both lines was not significantly different. Taken together, these results suggest that the phenotype of AS11 is not caused by a mutation directly affecting the integrity of the elongase complex or the supply of malonyl-CoA.

Desaturase activities, measured by following the conversion of ^{14}C- labeled 18:1 to 18:2 and 18:3, were also about 2-fold higher in AS11 than in WT homogenates. However, the proportion of labeled 18:2/18:3 produced was essentially identical, and, more important, the ratio of desaturase to elongase activity was about 3:1 in both the AS11 and WT lines. Northern analyses were performed to determine Δ15 desaturase and Δ12 desaturase transcript levels in green AS11 and WT seeds at mid-development (Fig. 3). The results indicated that there was no increased Δ15 or Δ12 desaturase mRNA in the AS11 mutant. Based on these findings, it is unlikely that the high 18:3/low 20:1 plus 18:1 fatty acid profile of AS11 is caused by a relative up-regulation of extraplastidic desaturase gene expression as has been suggested for mutant JB11 (Lemieux et al., 1990).

The KAS II activities of the AS11 and WT lines were identical (Table I). Thus, the small but significant change in 16:0/18:0 ratios found in AS11 was not explained by a lesion at this biochemical step.

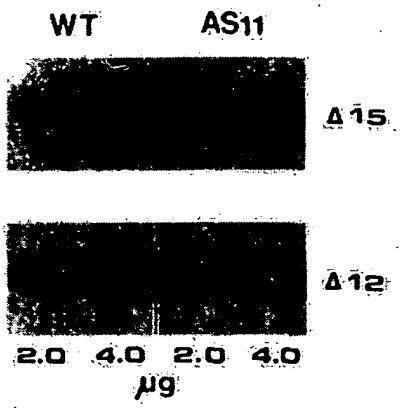

Figure 3. Northern analyses of transcripts for Δ15 and Δ12 desaturases in WT and mutant (AS11) seeds of *A. thaliana* at mid-development. Total RNA was isolated from *A. thaliana* WT and AS11 green seeds and blots were prepared as described in "Materials and Methods." The amount of RNA per lane was verified by staining the gel with ethidium bromide. The probe for Δ15 desaturase was the 1.4-kb *fad3* sequence from *B. napus*. The probe for Δ12 desaturase was the 1.4-kb *fad2* sequence from *A. thaliana*. Both probes were random-primer labeled.

Collectively, the data suggested that the primary biochemical lesion was not at the level of fatty acid biosynthesis. However, the observations of a delayed development in AS11 and lower overall lipid content in mature AS11 seed prompted us to perform more detailed studies of TAG biosynthesis in this mutant. Previous studies in *A. thaliana* and other members of the Brassicaceae had demonstrated that VLCFAs are biosynthesized by successive condensations of malonyl-CoA with 18:1-CoA or 18:0-CoA in the presence of reductant, whereas 18:1-CoA is desaturated to 18:2 and 18:3 while esterified to PC (Agrawal and Stumpf, 1985; Stymne and Stobart, 1987; Fehling et al., 1990; Taylor et al., 1991, 1992a; Kunst et al., 1992b). Then, as in other oilseeds, fatty acyl-CoAs are assembled into glycerolipids via the G-3-P (Kennedy) pathway (Kennedy, 1961; Barron and Stumpf, 1962; Stymne and Stobart, 1987; Kunst et al., 1992b; Taylor et al., 1992c; Taylor and Weber, 1994).

GC analyses of the neutral lipids (primarily TAGs and DAGs) in mature AS11 and WT seeds revealed a reduced

Table I. *Comparison of key fatty acid synthesis and modification enzyme activities in WT versus AS11 A. thaliana seed at mid-development*
Assays were conducted as described in "Materials and Methods." Activities reported ± SD (n = 2–4).

Enzyme(s) Assayed	Enzyme Activity in Vitro		
	WT	AS11	AS11 activity as percent of WT
	pmol min^{-1} mg^{-1} protein		
Elongase	135 ± 14	255 ± 12	190
Desaturase			
Total	410 ± 20	730 ± 40	180
% distribution			
[^{14}C]18:2	83.0	84.7	
[^{14}C]18:3	17.0	15.2	
Desaturase/elongase	3.0 ± 0.4	2.9 ± 0.3	n.s.d.[a]
ACCase	1520 ± 100	1380 ± 90	n.s.d.[a]
β KAS II	1.54 ± 0.10	1.58 ± 0.04	n.s.d.[a]

[a] n.s.d., Not significantly different.

TAG level in AS11 and, more important, a qualitative change in the acyl composition of the TAGs that accumulated. The TAG species present in mature seed were confirmed by NH_4^+-Cl MS/MS using the method described by Taylor et al. (1995) (data not shown). The shift in the $[M + 18]^+$ peaks for all major TAG species generally reflected an increase in the 18:3 content in AS11, relative to WT, in which 18:2 predominated. Of particular note, a set of C_{56} ($[M + 18]^+ = 927$) and C_{58} ($[M + 18]^+ = 957$) TAGs containing one or two eicosenoyl moieties, respectively, which were prevalent in WT, were less intense in AS11, whereas the relative intensity of C_{52} ($[M + 18]^+ = 871$) and C_{54} ($[M + 18]^+ = 895$) TAGs, containing two or three C_{18} fatty acids, respectively, increased in AS11.

The DAG pool in mature AS11 was highly elevated, representing 8 to 12% of the total lipid fraction, whereas in WT, it was characteristically about 1% or less (Fig. 4). This suggested the possibility that the rate of conversion of DAG to TAG is limiting in AS11. Although all DAG species were increased in AS11, the proportion of DAGs containing C_{20} moieties was most affected.

An examination of the fatty acid composition of AS11 and WT seed lipids throughout development indicated that, even at the early milky stage, the accumulation of all fatty acids was decreased in AS11 (Fig. 5A). However, it is evident that the synthesis of VLCFAs was more dramatically affected than synthesis of 18:2 and 18:3 at this early stage. This supports the acetate-feeding data, discussed previously (compare Fig. 2B), which indicated that by mid-development the elongation of 18:1 to 20:1 diminished

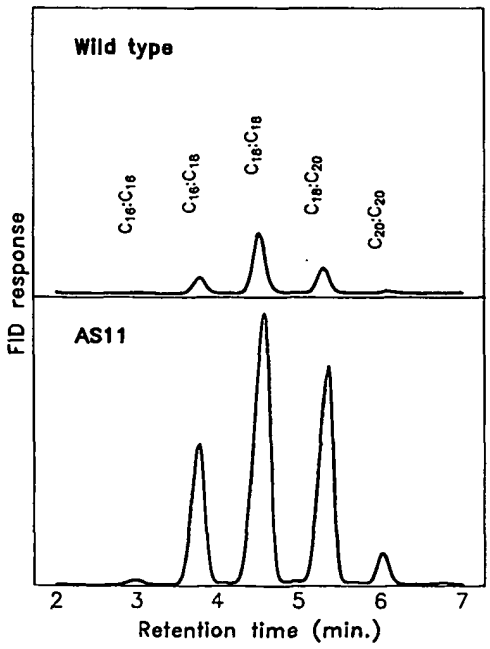

Figure 4. Comparison of WT and AS11 mature seed DAGs by GC. DAG fractions were isolated from equivalent dry weights of mature seed and purified and analyzed by GC as described in "Materials and Methods." Assignments of major DAG species were based on retention times relative to authentic standards. The internal standard (dierucin) is not shown.

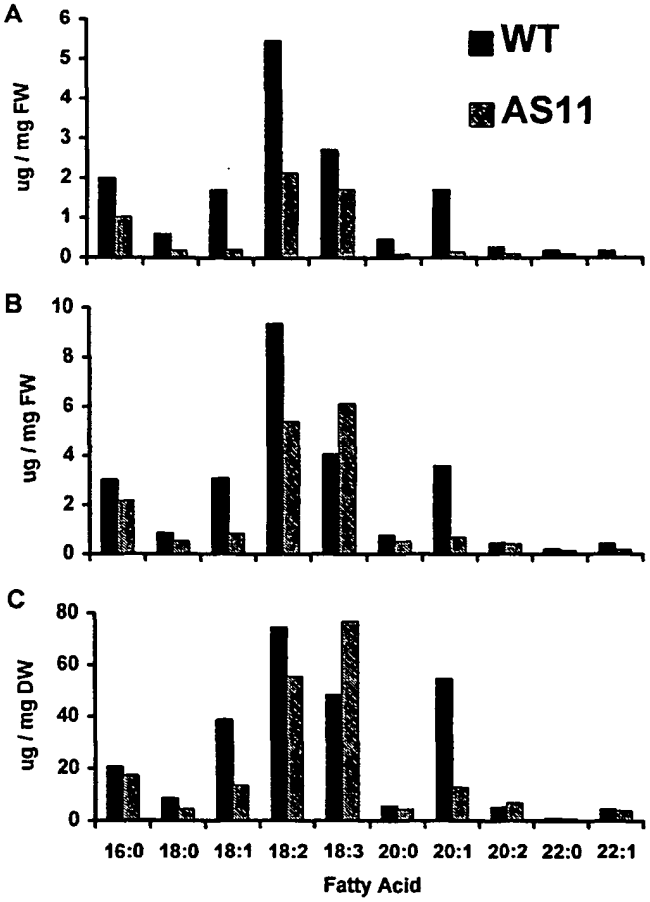

Figure 5. Fatty acyl content of WT and AS11 seed lipids at the milky (A), green (B), and mature (C) stages of development.

before desaturation of 18:1 to 18:3 had increased substantially. By the time maximum TAG deposition began in AS11 at 21 d.p.a., the acyl pool was dramatically different from that of WT seed and reflected a larger concentration of 18:3, relative to 20:1 and 18:1 (Fig. 5B). The result was that at maturity, in AS11, the levels of 20:1 and 18:1 in TAGs were decreased, whereas the level of 18:3 was increased relative to WT (Fig. 5C).

The relative proportions of the Kennedy pathway intermediates LPA, PA, and DAG, and the product TAG, as well as PC, were examined in seeds of WT and AS11 at the milky, green, and mature stages of development. At all three stages, the ratio of DAG/LPA plus PA and DAG/PC increased, whereas TAG/DAG decreased in AS11 relative to WT. This trend was readily evident in green developing seed (Fig. 6A) and was equally reflected in the ratios of lipid species produced by intact seeds at this developmental stage, during labeling with [^{14}C]acetate (Fig. 6B). There was a concomitant increase in all fatty acids in the DAG pool of AS11 seeds at mid-development, and, to a lesser extent, there was an associated backup of fatty acids into the PC pool (data not shown). TAG accumulation lagged throughout development in AS11, and this was evident even at the earliest milky stage. The TAG/DAG ratio in AS11 was consistently 3- to 5-fold lower than in WT at each

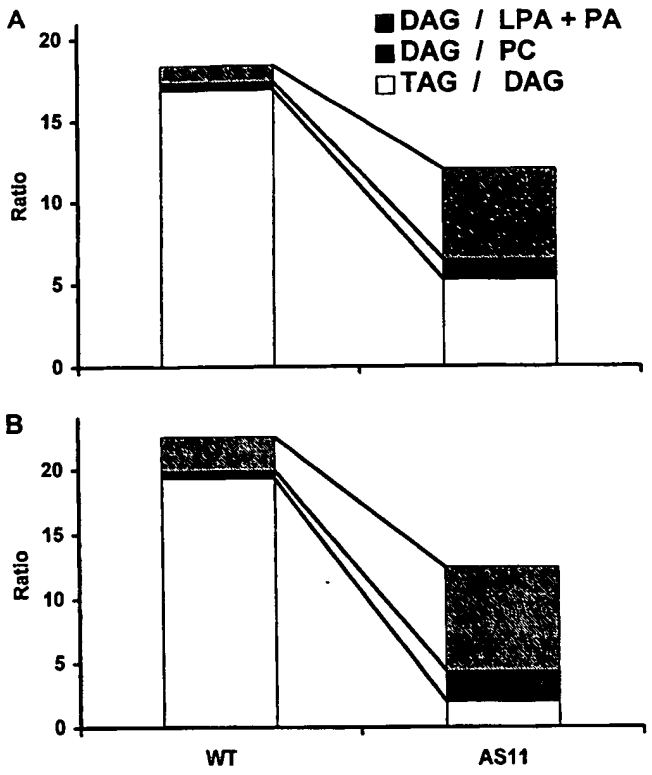

Figure 6. A, Relative proportions of lipid species present in WT and AS11 green developing seeds. Ratios were derived by comparing the levels of all Kennedy pathway intermediates on a μg/mg fresh weight basis. B, Relative proportions of radiolabeled lipid species synthesized during an 18-h incubation of WT and AS11 green seeds at mid-development with [1-^{14}C]sodium acetate as described in "Materials and Methods." Ratios were calculated by comparing the levels of all radiolabeled Kennedy pathway intermediates, TAG and PC, on a dpm/mg fresh weight basis.

developmental stage tested (Table II). This finding, combined with the increase in the levels of DAG relative to other Kennedy pathway intermediates (Fig. 6), strongly suggested that AS11 possessed a lesion that resulted in decreased DGAT activity.

This was confirmed by an in vitro study in which homogenates from WT and AS11 green seeds at mid-development were compared for their ability to incorporate [^{14}C]18:1-CoA into glycerolipids in the presence of G-3-P. 18:1-CoA was chosen as the acyl donor because previous stereospecific analyses of WT *A. thaliana* had shown that 18:1 was incorporated into all three *sn* positions in about equal proportions (*sn*-1, 30%; *sn*-2, 37%; *sn*-3, 33%), suggesting that the Kennedy pathway acyltransferases had similar affinities for 18:1-CoA (Taylor et al., 1995). Labeled DAG/LPA plus PA and DAG/PC ratios remained essentially unchanged between WT and AS11, indicating that the activities of the Kennedy pathway enzymes GPAT, LPA acyltransferase (EC 2.3.1.51), and PA phosphatase (EC 3.1.3.4), as well as CDP-choline:*sn*-1,2-diacylglycerol choline phosphotransferase (EC 2.7.8.2), were similar in the WT and AS11 homogenates. However, the ratio of labeled TAG/DAG was strongly reduced in the AS11 homogenate, suggesting that the DGAT activity was decreased (Fig. 7). Similar differences were observed when [^{14}C]20:1-CoA and G-3-P were supplied to AS11 and WT homogenates (data not shown).

A detailed stereospecific analysis of the TAGs from mature AS11 and WT seeds indicated that the composition at the *sn*-3 position was significantly altered in AS11 (Table III). In particular, relative to WT, there were dramatic decreases in the proportions of 18:1 and 20:1 and increases in the proportions of 18:2 and 18:3 at the *sn*-3 position in AS11 TAGs. These changes generally reflect the major differences in fatty acid content observed in mature seed TAGs (Figs. 1 and 5C) and indicate that the conversion of DAGs to TAGs is affected in AS11.

Direct DGAT assays were conducted using 1,2-diolein as an acyl acceptor and [^{14}C]18:1-CoA as the acyl donor. The results confirmed that, throughout development, the AS11 mutant had much reduced DGAT activity, which was correlated with a decreased TAG and increased DAG content (lower TAG/DAG ratio), compared to WT (Table II). However, in both WT and AS11 homogenates prepared from green seeds at mid-development, the DGAT activity was considerably higher when assayed in the presence of 20:1-

Table II. *Ratio of accumulating TAG/DAG and DGAT activities in WT and AS11 seed at various stages of development*

Developing seeds of WT and AS11 *A. thaliana* were harvested and total lipid extracts or homogenates were prepared. The relative amounts of TAG and DAG were determined as described in "Materials and Methods." DGAT activity in homogenates was assayed in the presence of 200 μM 1,2-diolein and 25 μM [1-^{14}C]18:1-CoA, with measurement of radiolabeled TAG species conducted as described by Weselake et al. (1991). SD ≤ 5% in all cases (n = 2).

Developmental Stage	TAG/DAG Ratio		DGAT Activity	
	WT	AS11	WT	AS11
			pmol min^{-1} mg^{-1} protein	
Milky	1.8	0.6	5.43	2.39
Early Green	n.d.[a]	n.d.	7.05	2.15
Green	16.9	5.3	6.80	2.00
Green-Brown	n.d.	n.d.	0.96	0.55
Mature	91.0	19.0	n.d.	n.d.

[a] n.d., Not determined.

Table III. *Stereospecific analyses of the TAG fraction of mature WT and AS11 seed of A. thaliana*

Seed	Percent Distribution of Each Fatty Acid over All *sn* Positions									
	16:0	18:0	18:1c	18:2	18:3	20:0	20:1	20:2	22:0	22:1
WT										
sn-1	31	21	30	32	38	24.5	35.5	63	19	45.5
sn-2	17	20	37	59.5	50	8.5	5.5	9	20	6.5
sn-3	52	59	33	8.5	12	67	59	28	61	48
AS11										
sn-1	50	34	34	23	21	41	60	74	30	26
sn-2	12.5	19	56	46	41	7	13	16	12.5	5.5
sn-3	37.5	47	10	31	39	52	27	10	57.5	68.5

CoA or 22:1-CoA as compared to 18:1-CoA or 18:2-CoA (data not shown). In competition studies, the DGAT in homogenates from both WT and AS11 exhibited a selectivity for 20:1-CoA over 18:2-CoA, incorporating 20:1 into TAGs at rates greater than 2-fold higher than 18:2. Thus, although DGAT activity was lower in AS11, the preference for incorporating very long-chain acyl-CoAs over polyunsaturated fatty acyl-CoAs in vitro was not altered. This suggests that the shift in *sn*-3 acyl composition observed in the mature seed TAGs of AS11 (Table III) is the cumulative result of reduced DGAT activity throughout development (Table II) and its effect on VLCFA biosynthesis (Fig. 5).

In summary, the data indicate that the EMS-induced lesion in a single nuclear gene in AS11 affects the conversion of DAG to TAG throughout seed development. Although the FAME phenotype would seem to suggest a lesion in the elongase complex or an up-regulation of desaturation, there is no biochemical evidence of a lesion directly affecting the integrity of the elongase or desaturase enzyme systems, although, ultimately, the fatty acid composition of seed TAGs is dramatically affected. We propose that the mutation in AS11 results in a decreased DGAT activity, such that TAG biosynthesis is impeded (locus affected by the mutation is designated *TAG*). Since 20:1 is normally incorporated preferentially into the *sn*-3 position by DGAT (see Table III), in AS11 there may be an accumulation of unesterified 20:1-CoA, which could feedback inhibit 18:1 elongation. This appears to lead to increased conversion of 18:1 to 18:2 and 18:3. By the time maximum lipid deposition begins at about 21 d.p.a. in AS11, the acyl-CoA pool available to DGAT is already enriched in 18:3, to the detriment of 18:1 and 20:1 (see Fig. 5B). Combined with a lower DGAT activity throughout seed development, the result is that at maturity, AS11 has reduced TAG levels containing proportionally less 18:1 and 20:1 and more 18:3 compared to WT. Although the amounts of most VLCFAs are depressed during development in AS11 as compared to WT (compare Fig. 5, A and B), the effect is most conspicuous by the decrease in 20:1, normally the predominant VLCFA in *A. thaliana*. An exception to this trend is 20:2, the product of 20:1 desaturation, which is present in higher proportions in mature AS11 than in WT (Figs. 1 and 5C). This may primarily reflect the fact that, since DGAT activity is reduced, some 20:1 is available for desaturation. The 20:2 is preferentially inserted into the *sn*-1 position (Table III), presumably by GPAT, displaying a most atypical distribution pattern for a VLCFA in the Brassicaceae (Taylor et al., 1995).

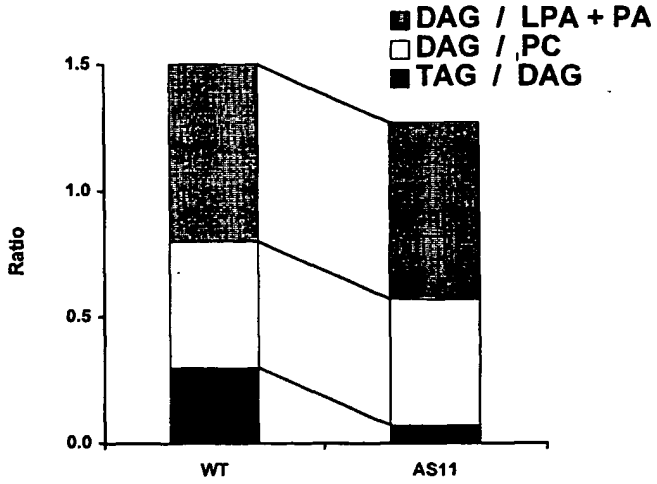

Figure 7. Relative proportions of glycerolipids synthesized in vitro during incubation of [1-^{14}C]18:1-CoA and G-3-P with homogenates prepared from WT and AS11 green developing seed. Ratios were calculated by comparing the levels of [1-^{14}C] 18:1 incorporated into Kennedy pathway intermediates, TAG and PC, expressed on a pmol min^{-1} mg^{-1} protein basis.

CONCLUSIONS

To our knowledge, the present study has shown for the first time in *A. thaliana* that reduced DGAT activity can strongly affect seed development and the pattern of fatty acid biosynthesis. In AS11, a decreased DGAT activity is correlated with delayed seed development, a reduced TAG content, and a repression of VLCFA biosynthesis with an accumulation of 18:3 in TAGs. These results suggest that overexpression of DGAT activity earlier in development may provide a means for channeling more carbon into VLCFAs and, ultimately, into TAGs. Such indirect regulatory mechanisms may have important implications for efforts to maximize the VLCFA content of other members of the Brassicaceae through biotechnology (Taylor et al., 1992b). The effects of the AS11 mutation on DGAT activity throughout development and on the acyl composition and amount of TAG that accumulates are unequivocal. However, it is not yet clear whether the lesion resulting in

decreased DGAT activity is in the DGAT gene itself or in a gene encoding a regulatory factor that directly controls DGAT. Alternatively, it is possible that the lesion in AS11 is in a gene regulating seed development, which results in pleiotropic effects on DGAT activity, fatty acid modification, and TAG bioassembly. Cloning the gene identified by the mutation in AS11, using chromosome walking (Kunst et al., 1992a, 1992b) or subtractive cloning techniques, will help to address these questions. High resolution RFLP mapping will reveal the RFLP marker closest to the gene identified by the AS11 mutation. This RFLP will serve as the starting point for chromosome walking, one of our future research goals.

Many researchers have reported EMS-induced mutations affecting seed and leaf fatty acid biosynthesis in *A. thaliana* (James and Dooner, 1990, 1991; Lemieux et al., 1990; Ohlrogge et al., 1991; Arondel et al., 1992; Browse and Miquel, 1992; Yadav et al., 1993). In an *A. thaliana* mutant with a deficiency in leaf chloroplast GPAT, the cytoplasmic (or ER) pathway was found to largely compensate for the deficiency in plastid GPAT, providing near-normal amounts of all of the lipids required for chloroplast membrane biogenesis (Kunst et al., 1988). That seminal investigation was important in providing a greater understanding of the regulation and interaction of the prokaryotic and eukaryotic pathways during leaf membrane lipid biosynthesis. However, to our knowledge, the current study is the first to document a mutation affecting the extraplastidic Kennedy pathway for storage lipid bioassembly in *A. thaliana* seeds. Our findings demonstrate the dangers inherent in characterizing complex seed lipid mutants based only on fatty acid composition obtained by a simple GC analysis, and we advocate a more rigorous application of analytical, biochemical, and genetic techniques in the analysis of EMS-induced mutants to identify strategic target genes in this model system. Indeed, it may be prudent to re-examine a number of mutant collections, heretofore characterized as fatty acid biosynthesis mutants (e.g. JB11 *ela1*; Lemieux et al., 1990) based solely on FAME analyses of seed lipids. It may be that some of these are, in fact, mutants with alterations to genes encoding other key lipid bioassembly (Kennedy) pathway enzymes or regulatory elements affecting their expression.

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The *Arabidopsis thaliana TAG1* mutant has a mutation in a diacylglycerol acyltransferase gene

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Summary

In *Arabidopsis thaliana* (ecotype Columbia) mutant line AS11, an EMS-induced mutation at a locus on chromosome II results in a reduced diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) activity, reduced seed triacylglycerol, an altered seed fatty acid composition, and delayed seed development. A mutation has been identified in AS11 in a gene, which we designated as *TAG1*, that encodes a protein with an amino acid sequence which is similar to a recently reported mammalian DGAT, and, to a lesser extent, to acyl CoA:cholesterol acyltransferases. Molecular analysis revealed that the mutant allele in AS11 has a 147 bp insertion located at the central region of intron 2. At the RNA level, an 81 bp insertion composed entirely of an exon 2 repeat was found in the transcript. While the seed triacylglycerol content is reduced by the lesion in AS11, there is no apparent effect on sterol ester content in the mutant seed. The *TAG1* cDNA was over-expressed in yeast, and its activity as a microsomal DGAT confirmed. Therefore, the *TAG1* locus encodes a diacylglycerol acyltransferase, and the insertion mutation in the *TAG1* gene in mutant AS11 results in its altered lipid phenotype.

Introduction

Plant seed oils are major sources of essential polyunsaturated fatty acids for human diets and renewable feedstocks for chemical industries. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids that are channelled into the cytosolic acyl CoA pool to sustain triacylglycerol accumulation. Triacylglycerol (TAG) biosynthesis is located in the endoplasmic reticulum, with glycerol-3-phosphate and fatty acyl CoAs as the primary substrates. There are three acyltransferases involved in plant storage lipid bioassembly, namely the glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), the *lyso*-phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51) and the diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). These three acyltransferases catalyse the stepwise acylation of the glycerol backbone, with the final step being the acylation of *sn*-1,2-diacylglycerol (DAG) by DGAT into TAGs, a biochemical process generally known as the Kennedy pathway (Stymne and Stobart, 1987). DGAT is the only enzyme in the Kennedy pathway that is exclusively committed to TAG biosynthesis. It has been suggested that DGAT may be one of the rate-limiting steps in plant storage lipid accumulation (Ichihara *et al.*, 1988) and thus a potential target in the genetic modification of plant lipid biosynthesis. However, this hypothesis has not been rigorously tested.

Among the three endoplasmic reticulum-based fatty acyl CoA acyltransferases, only the LPAT gene has been cloned from plants (Knutzon *et al.*, 1995; Lassner *et al.*, 1995). Like several other enzymes involved in storage lipid biosynthesis, acyltransferases are intrinsic ER proteins and are extremely difficult to purify. The research on plant DGAT has been largely limited to studies of activity profiles by using the particulate fractions generated by differential centrifugation of seed- or microspore-derived embryo homogenates (Weselake *et al.*, 1993). Although partial purification of DGAT from cotyledons of germinating soybean seeds was reported (Kwanyuan and Wilson, 1986), detailed molecular characterization of this enzyme is lacking.

We have previously reported the characterization of an EMS-induced *Arabidopis thaliana* mutant, AS11, with altered fatty acid composition (Katavic *et al.*, 1995). In comparison to wild-type plant seeds, AS11 seeds have reduced levels of the very long chain fatty acid eicosenoic acid (20:1) and reduced oleic acid (18:1) and accumulate α-linolenic acid (18:3) as the major fatty acid in triacylglycerols. The AS11 mutant has a consistently lower ratio of TAG/DAG in developing seeds, and accumulates an elevated amount of seed DAG, the substrate of diacylglycerol acyltransferase. Through a series of biochemical analyses, we showed that AS11 had reduced diacylglycerol acyltransferase activities throughout seed development. AS11 also has a reduced oil content phenotype, providing evidence that DGAT may be controlling flux into TAG biosynthesis. Genetic analysis indicated that the fatty acid phenotype is caused by a semi-dominant mutation in a nuclear gene, designated *TAG1*. The mutation was mapped to chromosome II, and was estimated to lie in the region

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approximately 17.5 ± 3 cM from the *sti* locus and 8 ± 2 cM from the *cp2* locus.

Here we report the identification, functional assignment and cloning of this diacylglycerol acyltransferase-related gene, *TAG1*, from *Arabidopsis thaliana* through a databank sequence search and assisted by map-based information. Our data demonstrate that the encoded product of the *TAG1* gene is homologous to the mammalian DGAT reported recently, and functions as a DGAT.

Results

Isolation of the TAG1 cDNA from Arabidopsis thaliana

Since one of the most likely defects in AS11 mutant is in the DGAT itself, we therefore attempted cloning strategies based on sequence information for enzymes that share common substrates with DGAT. One of the candidate enzymes that would serve this purpose is acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) (Chang *et al.*, 1997). Like DGAT, ACAT is an ER protein functioning as an *O*-acyltransferase by using acyl CoA as the fatty acyl donor for the esterification of free cholesterol to generate sterol esters. Through a BLAST database search, we identified an *Arabidopsis thaliana* expressed sequence tag (EST) (accession number AA042298) with a deduced amino acid sequence showing 41% identity to that of the yeast acyl CoA:cholesterol acyltransferase (Yang *et al.*, 1996; Yu *et al.*, 1996) within the short (104 amino acids) sequence that was available for the EST.

The corresponding cDNA (E6B2T7) clone was obtained from the *Arabidopsis* Biological Resource Center, Columbus, Ohio, USA. Upon complete sequencing, the 878 bp E6B2T7 clone was found to be a partial cDNA. However, the ORF prediction from this partial cDNA confirmed the initial EST search results in that the encoded product is structurally similar to ACAT, especially in the regions at the C-terminus. We were confident that the cDNA contained the 3′ untranslated region through an ORF search, although the polyA tail was missing.

We further used the partial cDNA sequence to search against *Arabidopsis thaliana* genomic sequence information. Subsequently, an *Arabidopsis* 'IGF' BAC clone 'F27F23' (accession number AC003058) was identified to include a region that matches the cDNA, and therefore it was concluded that this is the region encompassing the corresponding gene. Moreover, we were particularly encouraged by the fact that 'F27F23' is contained in the YAC clone, CIC06E08, which, according to the published map position (http://weeds.mgh.harvard.edu/goodman/c2_b.html), represents a region between cM 35.9 and 38.7 on chromosome II, similar to the location estimated for *TAG1* (Katavic *et al.*, 1995). In view of our previous results on the characterization of the AS11 mutant, the map position of this gene strongly suggested that it may encode a gene related to DGAT.

To clone a full-length cDNA, we designed a series of oligonucleotide primers based on the genomic sequences located at different positions 5′ upstream of the region covering the partial cDNA. We used these primers in combination with a primer located at the 3′ UTR of the partial cDNA (E6B2T7) to perform PCR reactions with cDNA phagemid prepared from an *Arabidopsis thaliana* (ecotype Columbia) silique-specific cDNA library (Giraudat *et al.*, 1992) as template. The longest cDNA amplified was 1904 bp, which we subsequently designated as *TAG1*, and deposited into Genbank under accession number AJ238008. We believe this cDNA represents a full-length clone because its size is in agreement with that of the transcript detected in the Northern blot (see below). The longest open reading frame is flanked by a 134 nt 5′ untranslated region and a 203 nt 3′ untranslated region. There is an in-frame stop codon (TGA at position nt 43) which is followed by an in-frame ATG at position nt 139. It is thus inferred that the ATG at position nt 139 is the initiation codon.

The primary structure of TAG1 predicts a DGAT-related enzyme

The predicted open reading frame of the *TAG1* cDNA encodes a polypeptide of 520 amino acids with a calculated molecular weight of 58 993 Da. Using the BLAST search program (Altschul *et al.*, 1990), it was found that the recently reported mouse diacylglycerol acyltransferase (accession number AF078752) (Cases *et al.*, 1998) showed the highest sequence similarity to the deduced amino acid sequence of *TAG1* (Figure 1a). TAG1 was also similar to a human acyl CoA:cholesterol acyltransferase-related enzyme (accession number AF059202). The human acyl CoA:cholesterol acyltransferase-related enzyme, also known as ARGP1, is most likely to be a DGAT with no significant ACAT activity, although the true nature of the enzyme awaits further confirmation (Oelkers *et al.*, 1998). The similarity between TAG1 and the mammalian DGAT extends over a region of more than 400 amino acids with a sequence identity of about 41%. A putative diacylglycerol/phorbol ester-binding motif, HKW-X-X-RH-X-Y-X-P, a signature sequence observed to be unique to DGAT while absent in the ACATs (Oelkers *et al.*, 1998), is located at amino acids 414–424 (Figure 1a). Among other cloned acyltransferases (e.g. GPATs, LPATs, dihydroxyacetone phosphate acyltransferases), it has been reported that there is an invariant proline in a highly hydrophobic block IV that may participate in acyl CoA binding (Lewin *et al.*, 1999). In the TAG1 sequence, the hydrophobic block from residues 221–229 containing an invariant proline at residue 224 might constitute such a motif.

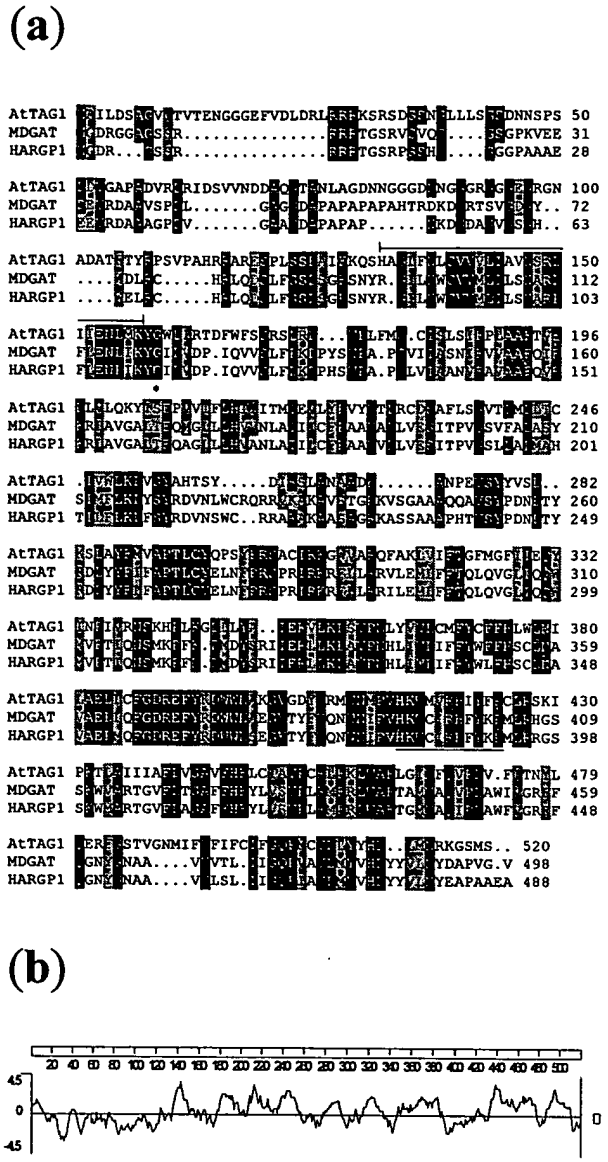

Figure 1. Analysis of TAG1 protein sequence.
(a) Comparison of TAG1 sequence and mammalian DGATs. AtTAG1, *A. thaliana* TAG1; MDGAT, mouse DGAT; HARGP1, human ARGP1 protein. Identical amino acid residues are highlighted in black. Conserved residues are shaded. The overlined segment indicates the insertion generated from the mutation in AS11. The putative diacylglycerol binding site is underlined. The asterisk denotes the SnRK1 potential targeting site.
(b) Kyte–Doolittle hydropathy plot of the TAG1 protein.

TAG1 showed some sequence similarity to other acyl CoA:cholesterol acyltransferases from a number of species (Chang *et al.*, 1997) (data not shown). However, this is largely confined to the *C*-terminus, and is significantly lower (around 30%) than the similarity of TAG1 to the mammalian DGAT.

The TAG1 protein has multiple hydrophobic domains (Figure 1b) and an analysis by the PC Gene program predicted that the protein has five possible transmembrane segments (amino acids 178–195, 233–253, 363–388, 433–476, 486–507). In the mammalian DGAT, a putative tyrosine phosphorylation motif was observed (Case *et al.*, 1998), but no apparent tyrosine phosphorylation site could be found in TAG1. However, a visual examination revealed a consensus sequence (X-L^{200}-X-K^{202}-X-X-S^{205}-X-X-X-V^{209}) identified as a targeting motif typical of members of the SnRK1 protein kinase family. The SnRK1 (SNF1-related protein kinase-1) proteins are a class of Ser/Thr protein kinases that have been increasingly implicated in the global regulation of carbon metabolism in plants (Halford and Hardie, 1998). Interestingly, similar SnRK1 targeting motifs could also be identified in the LPATs from coconut (Knutzon *et al.*, 1995) and meadowfoam (Lassner *et al.*, 1995), respectively.

The TAG1 *gene is ubiquitously expressed in* Arabidopsis

Northern blot analyses were performed to investigate the expression profile of the *TAG1* gene. Total RNA was extracted from different tissues, including roots, leaves, flowers, developing siliques, young seedlings and germinating seeds. We observed the highest steady-state level accumulation of *TAG1* transcripts in RNA isolated from germinating seeds and young seedlings (Figure 2a). *TAG1* transcripts were also detected in root, leaf and flower tissues, albeit at lower levels. Surprisingly, the *TAG1* gene is expressed in developing siliques at a level that is comparable to that of other vegetative tissues, but lower than that of germinating seeds and young seedlings. This expression profile in general is not inconsistent with the notion that DGAT is present in all plant tissues capable of TAG biosynthesis (Kwanyuan and Wilson, 1986). It has been shown in a number of plant species, including soybean and safflower, that germinating seeds actively synthesize TAGs (Ichihara and Noda, 1981; Kwanyuan and Wilson, 1986). The relatively high level of expression in roots is also consistent with the fact that root plastids are capable of synthesizing large amounts of fatty acids to sustain a very active TAG bioassembly process (Sparace *et al.*, 1992).

Southern blot hybridization was performed with genomic DNA digested with several restriction enzymes including *Bgl*II, *Eco*RI and *Hin*dIII. The *TAG1* gene has no internal *Bgl*II and *Hin*dIII sites, while one internal *Eco*RI site exists. Our Southern analysis suggested that *TAG1* most likely represents a single-copy gene in the *Arabidopsis* genome (Figure 2b).

An insertion mutation is found in the TAG1 *gene in mutant AS11*

Alignment of the genomic sequence (accession number AC003058) with that of the *Tag1* cDNA revealed that the

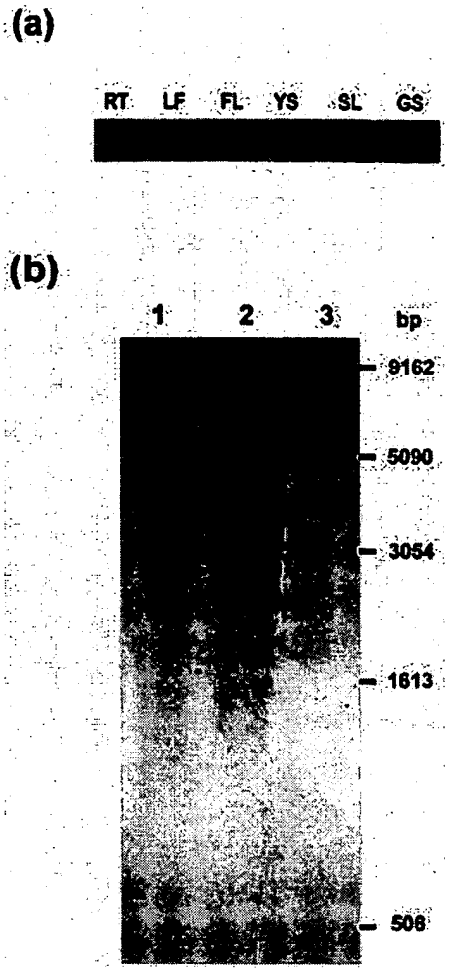

Figure 2. Northern and Southern analyses of the *TAG1* gene.
(a) Northern analysis of *TAG1* gene expression in *Arabidopsis thaliana*. Total RNA was extracted from roots (RT), leaves (LF), flowers (FL), young seedlings (YS), developing siliques (SL) and germinating seeds (GS).
(b) Southern blot analysis of the *TAG1* gene in *Arabidopsis thaliana*. Genomic DNA was digested with restriction enzymes *Bgl*II (lane 1), *Eco*RI (lane 2) and *Hind*III (lane 3). The *TAG1* DNA probe was ^{32}P-labelled by random priming.

TAG1 gene contains 16 exons and 15 introns, spanning a region of about 3.4 kb (Figure 3a). DNA containing the *TAG1* allele from AS11 was PCR-amplified and completely sequenced. The AS11 *TAG1* allele has a 147 bp insertion located at the central region of intron 2. The insertion is a duplication of a segment that is composed of 12 bp from the 3′ end of intron 1, the entire sequence of exon 2 (81 bp) and 54 bp from the 5′ end of intron 2.

In order to rule out the possibility of PCR artifacts, two sets of primers were used to perform further PCR amplifications. Primers A and B (see Experimental procedures) located in exons 1 and 3, respectively, amplified a DNA fragment that is about 150 bp longer from AS11 (Figure 3b, lane 2) than that from the wild-type (Figure 3b, lane 1). The second pair of primers, C and B (see Experimental procedures), with one to be found in both exon 2 and the insertion segment, and the other located in exon 3, generated two amplified fragments from AS11 (Figure 3b, lane 4) but only one from the wild-type (Figure 3b, lane 3). Hence these results confirmed that the insertion mutation we identified through sequencing reflected the true nature of the mutation in the *TAG1* gene in the AS11 genome.

The AS11 TAG1 *transcript has an 81 bp insertion in its open reading frame*

Northern blot analyses indicated that there was no difference in the expression profiles of the *TAG1* gene between the AS11 mutant and wild-type *A. thaliana* (data not shown). In order to investigate the effect of the mutation at the transcript level, reverse-transcription PCR (RT–PCR) was performed to amplify the *TAG1* transcript from RNA extracted from germinating seedlings of mutant AS11. Sequencing analysis revealed that there is an 81 bp insertion composed entirely of exon 2 in the transcript from AS11. The exon 2 in the repeat is properly spliced. The alteration of the transcript thus does not disturb the reading frame. However, this additional exon 2 sequence in the AS11 transcript would result in an altered DGAT protein with a 27 amino acid insertion (^{131}SHAGLFNLCVVVLIAVNSRLIIENLMK^{157}). As documented previously, there is a 40–70% reduction in DGAT activity throughout AS11 seed development (Katavic *et al.*, 1995). The 81 bp insert responsible for reduced DGAT activity in AS11 is visible in the comparison of RT–PCR products (compare Figure 3b, lane 5 (WT) and lane 6 (AS11).)

The TAG1 *gene insertion in* Arabidopsis *mutant AS11 affects seed triacylglycerol accumulation, but not sterol ester accumulation in seeds*

Because TAG1 also showed some sequence homology to ACATs from a number of species (Chang *et al.*, 1997), we decided to compare both triacylglycerol and sterol ester accumulation in seeds of the wild-type *A. thaliana* and AS11 mutant. While the triacylglycerol content and TAG/DAG ratios were reduced in AS11, consistent with our previously published findings (Katavic *et al.*, 1995), the proportions of sterol esters in WT and AS11 seeds were similar, at 0.8 and 1% of the total lipid extract, respectively (Table 1). If the *TAG1* lesion affected ACAT-like activity, one might expect a reduction in seed sterol esters, but this was not observed. These results indicated that TAG1 is not involved in sterol ester homeogenesis, and thus is not an acyl CoA:sterol acyltransferase.

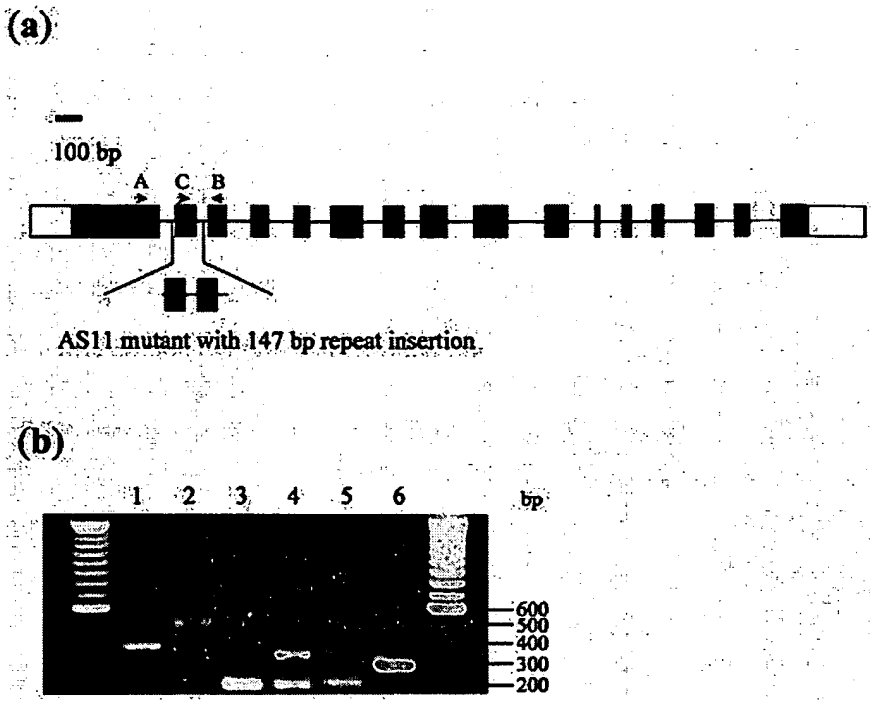

Figure 3. Analysis of the insertion mutation in the *TAG1* gene of AS11.
(a) Diagrammatic representation of the *TAG1* gene structure. The boxes indicate the 15 exons (solid boxes for coding regions, open box for untranslated regions), and the lines represent introns. A, B and C denote the positions of the primers used for PCR amplifications of the segments from wild-type (WT) and AS11.
(b) Gel separation of the PCR products amplified from wild-type (WT) and AS11. Lane 1, PCR product with primers A and B using WT genomic DNA as template. Lane 2, PCR product with primers A and B using AS11 genomic DNA as template. Lane 3, PCR product with primers C and B using WT genomic DNA as template. Lane 4, PCR product with primers C and B using AS11 genomic DNA as template. Lane 5, RT–PCR with primers A and B using RNA prepared from WT seedling RNA. Lane 6, RT–PCR with primers A and B using RNA prepared from AS11 seedling RNA.

Table 1. Comparison of AS11 (Katavic *et al.*, 1995) and wild-type *A. thaliana* seeds with respect to lipid profiles at mid-development, and the relative TAG, DAG and sterol ester contents in AS11 and WT seeds at maturity

Seed type	TAG/DAG ratio		TAG content at maturity		Sterol esters at maturity (% TLE)[d]
	Mid-development[a]	Maturity[a]	Relative[b]	Actual (nmol/mg DW)[c]	
WT	17	90	1.00	255	0.8
AS11	5	20	0.6	174	1.15

[a]Embryos staged and lipids measured as described in Katavic *et al.* (1995).
[b]Relative TAG content of 200-seed samples of AS11 and WT measured by magic angle sample spinning-^{1}H-NMR according to the method of Rutar (1989). The integration response for resonances attributable to liquid-like oil were summed and the value for AS11 seed is reported relative to the response for the WT control seed sample (the latter set at a value of 1.00).
[c]TAG content (nmol/mg dry weight) measured by transmethylation of a TLC-purified TAG fraction, followed by GC analysis of fatty acid methyl esters.
[d]Total lipid extract (TLE) was prepared as described by Taylor *et al.* (1991, 1992) and sterol esters isolated and characterized as described in Experimental procedures.

TAG1 *expression in yeast*

The *TAG1* cDNA over-expressed in yeast resulted in a 3.5–4-fold increase in microsomal DGAT activity compared to plasmid only (*pYES2*) control transformants, strongly suggesting that the *TAG1* gene product functions as a DGAT. This trend was identical in crude lysates (data not shown) and in microsomal fractions prepared by differential centrifugation (Figure 4). In contrast, when ^{14}C18:1-CoA was added to the yeast lysates, sterol esters were also labelled *in vitro* (data not shown), but there was no significant difference in the ^{14}C-labelled sterol esters produced by lysates from the *pYES2* Gal-induced control and the *pYES2:TAG1* Gal-induced transformant. This further suggests that the TAG1 product does *not* encode an acyl CoA:sterol acyltransferase (ACAT homologue).

Discussion

We have identified a gene and cloned its corresponding cDNA, which encodes a protein with significant primary structure similarity to mammalian DGAT. We concluded that this gene is the primary lesion in AS11 based on our previous genetic and biochemical data, physical

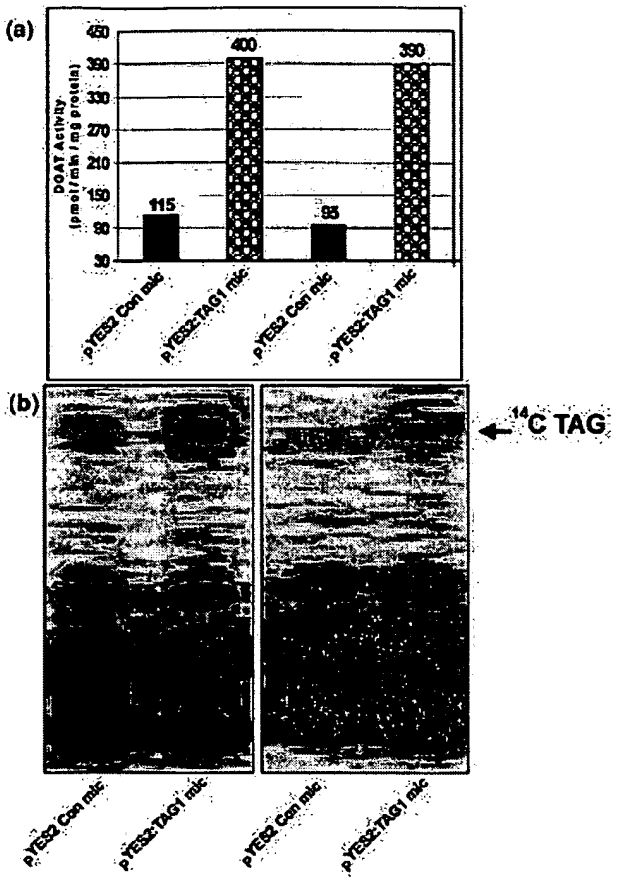

Figure 4. Expression of the *TAG1* cDNA in yeast.
Host cultures of strain YMN5 were transformed with *pYES2* plasmid only (*pYES2* Con; without *TAG1* insert) or with *pYES2* containing the *TAG1* cDNA insert (*pYES2:TAG1*). Following induction in the presence of galactose, transformants were lysed and assayed for DGAT activity as described in Experimental procedures.
(a) The results obtained in two separate DGAT activity experiments performed with microsomal fractions (pYES2Con mic versus pYES2:TAG1 mic) are shown.
(b) Corresponding radio-TLC scans of the microsomal DGAT assay reaction products. The spot intensities are presented on a logarithmic scale. Radiolabelled triacylglycerol bands are indicated by the arrow.

mapping information and gene expression profile, and therefore designated it as *TAG1*. Our previous data indicated that the *TAG1* locus in AS11 has a lesion affecting DGAT activity and consequently the amount and acyl composition of seed triacylglycerols (Katavic *et al.*, 1995). In this study, we have revealed that in AS11, there is an insertion mutation in a gene homologous to the mammalian DGAT that results in an extra exon 2 sequence in its transcript. The DNA aberration observed in this mutant was unexpected, since ethyl methanesulphonate (EMS) generally causes point mutations. Although we cannot rule out the possibility that this AS11 mutant was the result of a spontaneous mutation event, EMS-induced deletions and insertions have been reported in other systems (Mogami *et al.*, 1986; Okagaki *et al.*, 1991).

The present research allowed us to conclude that the encoded product of the *TAG1* gene functions as a diacylglycerol:acyl CoA acyltransferase. The sequence similarity between TAG1 and the mammalian DGAT as well as the putative diacylglycerol-binding motif favour the probability that TAG1 is a plant DGAT. This was confirmed by over-expression of TAG1 in yeast, and is in accordance with the biochemical defect we observed in AS11.

One could expect that a DGAT gene would be expressed highly in developing seeds, a stage where rapid TAG deposition takes place. Interestingly, the highest level of *TAG1* transcript we could detect was in germinating seeds and young seedlings. It has been shown that DGAT activities are present in various tissues, and the rate of triacylglycerol synthesis in green cotyledons is about half of that in developing seeds (Wilson and Kwanyuan, 1986). In fact, DGAT has been detected and partially purified from germinating soybean cotyledons (Kwanyuan and Wilson, 1986). The AS11 oil content is reduced by about 25–35% (Table 1), with no wrinkled-seed phenotype as described in other low-seed-oil mutants (Focks and Benning, 1998).

The nature of the mutation in *TAG1* would result in a structurally altered DGAT, and our Southern analysis suggested that *TAG1* represents a single-copy gene. Our biochemical data, which measured acyl CoA-dependent DGAT activity, showed that in AS11, there is a reduced DGAT activity (40–70% lower than WT), lower seed TAG and delayed seed development. However, some DGAT activity still remains in AS11, and, while seed development is delayed, significant TAG eventually accumulates. Thus, one possibility is that the lower DGAT activity in AS11 actually results in the delay of seed development. Alternatively, a relatively large proportion of the TAG that is synthesized in AS11 could come from another (possibly seed-specific) DGAT isoform, or from an alternative mechanism, such as diacylglycerol:diacylglycerol transacylase activity (Stobart *et al.*, 1997). It may be that, in the presence of a poorly functioning DGAT, DAG transacylase may play a far more important role than previously thought in catalysing TAG biosynthesis in seeds.

The fatty acyl composition of AS11 seed oil is enriched in 18:3 but greatly reduced in 20:1 and 18:1, and the lower DGAT activity in AS11 results in elevated DAG and reduced TAG levels. In a previous publication (Katavic *et al.*, 1995), we postulated that the reduced DGAT activity in AS11 resulted in repression of very long-chain fatty acid (e.g. 20:1) biosynthesis. However, given our current findings, it is also possible to speculate that, because DAG accumulates and has more time to equilibrate with phosphatidylcholine (via CDP–choline:*sn*-1,2 diacylglycerol choline phosphotransferase, EC 2.7.8.2), this could allow an enrichment in conversion of 18:1 to polyunsaturated C_{18} moieties while esterified to phosphatidylcholine. When combined with the enrichment of the acyl CoA pool with

polyunsaturated C_{18} acyl CoAs released from phosphatidylcholine (via exchange with 18:1-CoA catalysed by the reversible *lyso*-phosphatidylcholine acyltransferase (EC 2.3.1.23)), the end result would be increased 18:2 and 18:3 in both the acyl CoA pool and phosphatidylcholine and DAG pools, with less 18:1-CoA available for elongation. Such a scheme could also explain the acyl composition observed in AS11.

It is unclear why the *TAG1* is expressed highly during seed germination, a stage where lipolytic processes normally take place. It has been suggested that there is subcellular compartmentation of the synthetic and hydrolytic mechanisms associated with the TAG metabolism in germinating cotyledons (Wilson and Kwanyuan, 1986). Although *TAG1* is expressed at a lower level in siliques containing developing seeds, the protein may be very stable. In this context, it should be noted that the mammalian DGAT is expressed at the highest level in the small intestine where fat absorption occurs. In light of the putative protein kinase-targeting motif conserved between TAG1 and LPATs, it is also reasonable to assume that acyltransferase enzyme activities are also regulated at the protein level.

In conclusion, our collective biochemical and cloning and sequencing analysis data from the current and previous studies of mutant AS11, along with the current yeast expression data of the *TAG1* cDNA, suggest that *TAG1* encodes a DGAT isoform ubiquitously present in *A. thaliana*.

Experimental procedures

Plant material

Arabidopsis thaliana ecotype Columbia and mutant AS11 were grown under conditions described previously (Katavic *et al.*, 1995). The *A. thaliana* mutant line AS11 was generated and characterized relative to wild-type (WT) *A. thaliana* ecotype Columbia, as described by Katavic *et al.* (1995).

DNA manipulation

Standard methods and procedures were used for DNA preparation, plasmid propagation and isolation (Sambrook *et al.*, 1989). Sequencing was conducted on an Applied Biosystems Model 373A DNA Sequencing System using the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc.). The nucleotide and the deduced amino acid sequences were compared with sequences available in databanks using the BLAST program (Altschul *et al.*, 1990).

Southern and Northern analysis

Total RNA was extracted from different tissues at various developmental stages, using the method of Lindstrom and Vodkin (1991). RNA samples were denatured with formaldehyde and separated on 1.2% formaldehyde–agarose gels. About 5 μg of total RNA was loaded, and the amount of RNA per lane was calibrated by the ethidium bromide-staining intensity of the rRNA bands. Genomic DNA was isolated, digested with restriction enzymes, and a Southern blot analysis was performed according to Sambrook *et al.* (1989). The *TAG1* DNA probe was ^{32}P-labelled by random-priming according to the manufacturer's protocols (BRL).

PCR strategy

Primers used for the amplification of the *TAG1* gene were as follows: DGAT1 (AGACACGAATCCCATTCCCACCGA), DGAT2 (AGTGGTGACAACGCAGGGATGATG), DGAT3 (ATGGTCGCTCCCACATTGTGT), DGAT4 (CATACAATCCCCATGACATTTATCA). DGAT1 and DGAT2 amplify the 5′ half of the *TAG1* gene and DGAT3 and DGAT4 amplify the 3′ end of the *TAG1* gene. Genomic DNA from AS11 was used as template for PCR amplification of the mutant *TAG1* allele using the thermal profile: 94°C for 3 min; 40 cycles of 94°C for 30 sec, 62°C for 45 sec, 72°C for 1 min; and finally 72°C for 15 min. To further confirm the mutation, primers A (CGACCGTCGGTTCCAGCTCATCGG), B (GCGGCCAATCTCGCAGCGATCTTG) and C (TAAACAGTAGACTCATCATCG) were used in pairs (A and B; B and C) to amplify the internal fragment containing the mutation. The primers DGAT1 and DGAT4 were used for PCR amplification of the cDNA with an *A. thaliana* silique cDNA library as template. Primers A and B are also used in RT–PCR amplification of the cDNA fragment encompassing the insertion segment.

Construction of TAG1 *multi-copy vector and transformation and characterization of DGAT expression in yeast*

The *TAG1* cDNA was cloned into pBluescript SK as described (Hadjeb and Berkowitz, 1996). The cDNA was cut out from the vector with *KpnI*/*XbaI*, and subsquently cloned into the respective sites of the yeast expression vector pYes2 (Invitrogen). The construct was confirmed by sequencing. Constructs with *Tag1* transcription under the control of the *GAL1* promoter released a fragment of approximately 1.9 kb. Because the *TAG1* fragment has its own initiating ATG codon, the product expressed is not a fusion protein. As a host for yeast expression, an *SLC* deletion strain (YMN5 [*slc1Δ2::LEU2 ura3*]) (kindly provided by M.M. Nagiec and R.C. Dickson, University of Kentucky, Lexington, Kentucky. USA; Nagiec *et al.*, 1993) was used; we reasoned that in this mutant, the endogenous DAG pool may be lower than in WT yeast, and that this would allow us to maximize the activity from over-expressed TAG1 in the presence of exogenously supplied ^{14}C-DAG during *in vitro* DGAT assays of transformant lysates. Yeast transformation was performed according to Elble (1992). YMN5 transformants containing vector only (*pYES2*) were used as controls. Single colonies were cultured overnight in 10 ml of SD medium (synthetic dextrose medium with glucose and without uracil, as described by Ausubel *et al.*, 1995; vol. 2, p. 13.1.3) on a rotary shaker (270 rev/min) at 28°C. The next day, 1 ml of the overnight culture was used to inoculate 99 ml of SD medium. Cells were grown at 28°C for about 17 h. Then 50 ml of this culture were added to 200 ml of SD medium and cells were grown for 4 h. Cells were pelleted, washed once with cold water, and resuspended in 250 ml of medium for induction of expression (SD medium containing 2% galactose (Gal) and without uracil). Cells were reincubated at 28°C, with shaking at 270 rev/min, and

harvested after 4 h. Gal-induced yeast transformants were harvested by centrifugation at 12 500 *g* for 15 min and resuspended in ice cold 100 mM HEPES–NaOH, pH 7.4, containing 1 mM EDTA and 1 mM DTT. All further procedures were carried out at 4°C. Cell lysates were prepared using acid-washed glass beads as described by Ausubel *et al.* (1995). Microsomes were prepared from yeast transformant lysates by differential centrifugation followed by resuspension of the 10 000 *g*–100 000 *g* pellet fraction in grinding medium as described previously (Taylor *et al.*, 1991). Protein in yeast lysates and microsomal fractions were dispersed by sonicating fractions on ice for 2 min in 30 sec cycles with a Labsonic 2000U probe sonicator on the low setting (B. Braun Biotech Inc., Allentown, Pennsylvania, USA). Protein concentrations were measured using the Bradford (1976) assay, protein levels in each fraction were normalized, and equivalent aliquots (250 µg protein) were assayed for DGAT activity as described below.

Lipid substrates and DGAT analyses

^{14}C-labelled diolein [1-^{14}C oleic] (specific activity 55 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals (St Louis, Missouri, USA). The ^{14}C-labelled *sn*-1,2-diolein isomer was purified by TLC on borate-impregnated plates and emulsified in HEPES buffer in the presence of 0.2% Tween-20 as described by Taylor *et al.* (1991). 20:1-CoA, CoASH, ATP and all other biochemicals were purchased from Sigma.

DGAT assays were conducted at pH 7.4, with shaking at 100 rev/min in a water bath at 30°C for 30–60 min. Assay mixtures (0.5 ml final volume) contained lysate or microsomal protein (250 µg), 90 mM HEPES–NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1 mM $MgCl_2$, 200 µM *sn*-1,2 diolein [1-^{14}C oleic] (specific activity 2 nCi/nmol) in 0.02% Tween-20, and 18 µM 20:1-CoA as the acyl donor. The ^{14}C-labelled TAGs were isolated by TLC on silica gel G plates developed in hexane:diethyl ether:acetic acid (70:30:1 v/v/v/), the radiolabelled TAG bands visualized on a Bioscan AR-2000 radio-TLC scanner using Win-Scan 2D© software (Bioscan Inc., Washington DC, USA) and the bands scraped and quantified as described by Taylor *et al.* (1991).

Further lipid and sterol ester analyses in AS11 and WT

Total lipid extracts (TLEs) and lipid class analyses in WT and the AS11 mutant were performed as described by Taylor *et al.* (1991, 1992) and Katavic *et al.* (1995). Relative seed oil content was also measured by magic angle sample spinning ^{1}H-NMR, according to the method of Rutar (1989). Analyses were conducted with 200-seed samples of intact wild-type and AS11 seeds using a Bruker AM wide-bore spectrometer (Bruker Analytische Masstechnik GmbH, Karlsruhe, Germany) operating at 360 MHz. To reduce anisotropic line broadening, the seed sample was rotated at 1 kHz in a zirconium rotor oriented 54.7° to the magnetic field. The integration responses for resonances attributable to liquid-like oil were summed and the value for AS11 seed was recorded relative to the response for the WT control seed sample, the latter set at a value of 1.00.

Sterol esters were purified from the TLEs by thin layer chromatography (TLC) on silica H plates developed in hexane:-diethyl ether:formic acid (80:20:2, v/v/v). After elution from the silica H with chloroform:methanol (2:1, v/v), the sterol esters were quantified by saponification followed by methylation of the resulting fatty acids with 3 N methanolic HCl. The fatty acid methyl esters (FAMEs) were analysed by GC as described previously (Taylor *et al.*, 1991). The free sterols released by saponification were also analysed by GC on a 30 m DB-5 column; GC temperature program: initial temperature 180°C, increasing at 10°C min^{-1} to 300°C and held at this temperature for 15 min. The sterol ester content was reported as a percentage of the TLE, i.e. FAMEs released from sterol esters calculated as proportion of the FAMEs released by transmethylation of the total lipid extract.

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GenBank, DDBJ and EMBL mRNA nucleotide sequence database accession number AJ238008 (*TAG1* sequence data).

Note added in proof

Whilst a revision to this manuscript after its review was in progress, another paper describing *A. thaliana* DGAT was published (Hobbs *et al.* (1999) *FEBS Lett.* **452**, 145–149). The two ORFs are identical and Hobbs *et al.* have shown DGAT activity of the ORF product in a recombinant insect cell line culture.

RELATED PROCEEDINGS APPENDIX

None